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**PCT** WORLD INTELLECTUAL PROI International I

INTERNATIONAL APPLICATION PUBLISHED UNDE WO 9606630A1

(51) International Patent Classification 6 : A61K 38/08, 38/16, 39/00, 39/008, C07K 7/06, 14/44, G01N 33/564, 33/566 — A1

(11) International Publication Number: **WO 96/06630**

(43) International Publication Date: 7 March 1996 (07.03.96)

(21) International Application Number: PCT/US94/09795

(22) International Filing Date: 26 August 1994 (26.08.94)

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(81) Designated States: CA, JP, US.

Published
*With international search report.*

(54) Title: METHODS AND COMPOSITIONS FOR MODULATING A T CELL RESPONSE

(57) Abstract

T cell modulatory peptides, methods for identifying such peptides and methods of use therefor are provided in the present invention. In one embodiment T cell modulatory peptides for a specific antigen are identified which stimulate interferon-gamma secreting Th1 cells and which have a high MHC binding affinity; these modulatory peptides can be administered to a subject to modulate, for example, allergic disorders. In another embodiment T-cell modulatory peptides for a specific antigen are identified which stimulate IL-4 secreting Th2 cells and which have a low MHC binding affinity; these modulatory peptides can be administered to a subject to modulate, for example, autoimmune disorders.

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# METHODS AND COMPOSITIONS FOR MODULATING A T CELL RESPONSE

## BACKGROUND OF THE INVENTION

1. *Field of the Invention*

The present invention relates generally to the T cell response and specifically to methods and compositions for modulating the Th1 and Th2 response to a specific antigen.

2. *Description of Related Art*

Although the immune response is often seen as beneficial, in certain circumstances the immune response to an antigen can actually be harmful to the animal in which the immune response occurs. An example where the immune response creates a condition wherein the host is subject to serious pathologic sequelae is in such autoimmune diseases as lupus erythematosus. In lupus erythematosus, antibodies are often present which react with determinants in the thyroid, erythrocytes, DNA, and platelets of the host. Consequently, for autoimmune disease it has traditionally been desirable to suppress the immune response.

Another example where the suppression from immune response would be desired is in the treatment of allergies. It has been established that IgE antibodies against allergens cause hay fever, and are involved in the other allergic diseases such as extrinsic asthma. The crucial role of IgE antibodies in the allergic diseases raised the possibility that the regulation and suppression of the IgE antibody formation against allergens would be one of the fundamental treatments for allergic diseases.

The immune system has the potential to make a variety of responses and, as noted above, the response is sometimes deleterious. A correct immune response to an infectious agent is dependent on the activation of an appropriate set of immune effector functions, both cell-mediated and humoral.

Two parallel T cell subsets, one that bears the CD4 receptor ($CD4^+$) and another that bears the CD8 receptor ($CD8^+$) have been identified. These subsets are specialized to recognize different antigen presenting MHC molecules, CD4+ T cells recognizing class II MHC molecules and CD8+ T cells recognizing class I MHC molecules.

The CD4+ subset can be further subdivided into subpopulations, termed Th1 and Th2, which secrete different patterns of lympho/cytokines and also reciprocally modify each other's effector functions. The cytokines are molecules which affect other cells and are responsible for much of the effector function of the different subpopulations. Th1 cells secrete interferon $\gamma$ (IFN-$\gamma$), interleukin 2 (IL-2) as well as tumor necrosis factor (TNF) and Th2 cells secrete interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin-9 (IL-9), interleukin 10 (IL-10), and interleukin 13 (IL-13). A third subset phenotype (Th0), which secretes a combination of the cytokines characteristic of both subsets, has been reported and thought to be present early during an immune response. Recent studies have established the human equivalent for CD4+ T cells with cytokine pattern functions that are comparable to murine Th1 and Th2 cells (Romagnani, S., *Ann. Rev. Immunol.*, 12:227, 1994), as well as a group of cells in both species which appear to secrete both families of cytokines (Th0). The emerging consensus is that the Th1 and Th2 subsets arise as alternative progeny from a common precursor as a result of antigen activation and the presence of cytokines (Röcken, M., *et al., J. Immunol.*, 148:1031-1036, 1992; Seder, R., *et al., J. Exp. Med.*, 176:1091-1098, 1992).

The similarities in the general features of T cell regulation between mice and humans have made murine models exceptionally useful for understanding the corresponding human *in vivo* activities of lymphokines and consequences of modulating levels of T cell subsets *in vivo*.

There is now considerable evidence that the failure to control or resolve infectious diseases often results from inappropriate rather than insufficient immune responses. For example, the non-healing form of leishmaniasis in mice and humans, as well as lepromatous leprosy, represent strong but counterproductive Th2-dominated responses (Powrie, F., *et al.*, *Immunology Today*, 14:270-274, 1993). Thus intervention in these situations could be aimed at expanding Th1 responses or shifting the balance so as to enhance IFN-$\gamma$ production. Indeed, the presence of IL-12 has been shown to be effective in steering responses towards Th1 (Trinchieri, G., *Immunology Today*, 14:335-338, 1993).

Similarly, most allergic diseases appear to be dominated by Th2-like responses to environmental antigens. Th2 cells stimulate the production of three important features of allergic diseases; IL-4 induces IgE production, IL-5 is responsible for eosinophilia and the combination of IL-3, IL-4 and IL-10 leads to mast cell production (O'Hehir, R.E., *et al.*, *Annu. Rev. Immunol.*, 9:67, 1991) (cells which release their content of allergy-causing mediators upon stimulation with an allergen). On the other hand, Th1 cells mediate a very different set of inflammatory immune responses including delayed type hypersensitivity and some autoimmune responses. IFN-$\gamma$ and to some extent TNF activate macrophages which in turn have the ability to kill a wide variety of intracellular and extracellular pathogens. Therefore, intervention by upregulating Th1 responses or IFN-$\gamma$ production and down regulation of IL-4 secretion could be a key factor in therapeutic strategies. Indeed, IFN-$\alpha$, which shares many activities with IFN-$\gamma$, has been shown to reduce IgE and eosinophilia.

In contrast to the benefits of Th1-induced inflammation in responses to pathogenic microorganisms, these responses to autoantigens are usually deleterious. Although autoimmune diseases are generally multifactorial in humans, in most experimental models of human autoimmune conditions, for example, experimental allergic encephalomyelitis (EAE) a model for multiple sclerosis, has been shown to be primarily mediated by myelin basic protein (MBP)- specific Th1 cells (Powell, M.B., *et al., Int. Immunol.*, 2:539, 1990; Ruddle, N.H., *et al., J. Exp. Med.*, 172:1193, 1990). There is also evidence of Th1 cell involvement in insulin-dependent diabetes mellitus (IDDM) in humans and animal models (Tisch, R., *et al., Nature*, 366:72-75, 1993). Anti-IFN-$\gamma$ has been shown to protect against diabetes in non obese diabetic (NOD) mice. In this situation, a predominant Th2 response may be beneficial. Indeed, recently the presence of $CD45RC^{low}$ (in rat) and $CD45RB^{low}$ (in mouse) CD4+ cells, which predominantly secrete Th2-like cytokines, have been shown to be protective in autoimmunity (Powrie, F., *et al., J. Exp. Med.*, 179:589-600, 1994). This protection has clearly been shown to be mediated by IL-4.

Thus, the present invention fulfills a need to exploit the immune system itself to divert predominant T cell responses away from disease; diverting the response from the dangerous Th2 to the possibly protective or less dangerous Th1 in the case of allergic responses, as well as from the deleterious Th1 to Th2 in autoimmunity. Finally, deviations in cytokine production seen after altering ligand-receptor affinities are useful in achieving protective immune responses after vaccination. The present invention shows that rather than trying to prevent an autoimmune or an allergic response, the most successful strategy is instead to elicit another, diversionary and/or protective response.

## SUMMARY OF THE INVENTION

The present invention is based on the discovery that the ratio of subsets of T cells, Th1 to Th2, can be altered by stimulation of a subset, thereby providing a means for eliminating deleterious effects caused by elevated levels of one or the other of these cell populations. Th1 cell responses are often associated with autoimmune disorders, while Th2 responses are associated with allergic responses.

In one embodiment, the invention provides a method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IFN-$\gamma$ secreting T cell population, comprising preparing an array of variant immunodominant peptides from the antigen and selecting from the array, a peptide that has high MHC binding affinity and that induces proliferation of T cells specific for the antigen. Likewise in another embodiment, the method of the invention is utilized for identifying a peptide which stimulates an IL-4 secreting T cell population and the peptide has low MHC binding affinity.

In another embodiment, the invention provides a method of modulating a T cell response to a specific antigen in a subject with a T cell disorder, comprising administering to the subject an effective amount of a T cell modulatory peptide for the antigen, wherein the peptide stimulates an IFN-$\gamma$ or an IL-4 secreting T cell population.

In yet another embodiment, the invention provides T cell modulatory peptides.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows profiles of the determinant regions on the PSP molecule in BALB/c and B6 mice. Lymph node cells of four to six pooled BALB/c (A and B) or B6 (C and D) mice previously immunized with 2 μg of rPSP (A and C) or 2.5 μg of HPSP (B and D) were cultured in 28 μM solutions of each of 155 peptides for 72 hours and then pulsed for an additional 18 hours with 1 μCi of [$^3$H]thymidine. Peptides were 15mers advancing by three residues and cover the entire PSP molecule. Peptide number refers to the N-terminal residue of the 15mer. Data are expressed as [$^3$H]thymidine incorporation in individual cultures in FIGURES 1, 2 and 3. To assure reproducibility, experiments were performed two to four times.

FIGURE 2 shows fine determinant mapping of PSP determinant regions in BALB/c mice. Lymph node cells of individual BALB/c mice previously immunized with 2.5 μg of HPSP were cultured along with each peptide (at 14 μM) within determinant regions with cores 138-147/143-155 (A) and 369-376 (B) under the same conditions used in FIGURE 1. Peptides are 15mers advancing by one residue and cover selected determinate regions represented in FIGURE 1.

FIGURE 3 shows fine determinant mapping of PSP determinant regions in B6 mice. Lymph node cells of individual B6 previously immunized with 2.5 μg of HPSP were cultured along with each peptide (at 14 μM) within determinant regions with cores 138-147/143-155(A), 194-201(B), or 456-464 (C) under the same conditions used in FIGURE 1. Peptides are 15mers. advancing by one residue and cover selected determinant regions shown in FIGURE 1.

FIGURE 4 shows core sequences for determinant regions defined in FIGURE 2. The cores are defined according to the proliferation results form FIGURE 2 and include shared residues of peptides which induce at least 50% of the maximal proliferation for that determinant region. PSP peptide sequences are indicated by the single-letter amino acid code.

FIGURE 5 shows lymphokine profile for the major PSP determinant regions in BALB/c and B6. Lymph node cells ($5 \times 10^6$) from individual BALB/c (A. mice 1 and 2) and B6 mice (B mice 1 and 2) were cultured with peptides corresponding to determinant regions with cores 369-376 (BALB/c) and 456-464 (B6) in 1 ml of complete medium. Supernatants were collected after 48 hours centrifuged, stored at -70°C and thawed 10 minutes before assay for IFN-$\gamma$ and IL-4 by ELISA cultures were established as in FIGURE 2 and assayed for proliferation. The results express the lymphokine and proliferation profiles from two individual animals for both BALB/c and B6.

FIGURE 6 shows peptides within the determinant region with core 369-376 are able to induce Th2 cells in the presence of anti-IFN-$\gamma$ BALB/c mice were immunized with 2.5 $\mu$g of HPSP (A and B) and injected i.p. with 500 $\mu$g of XMG 1.2 antibody in PBS (B only) on days 1 and 3. On day 9, popliteal lymph node cells from three animals were pooled from each group and cultivated for 48 hours with peptides 364-378 (A and B) or 368-382 (A) in the presence or absence of 20 $\mu$g/m of antibody R46A2 (anti-IFN-$\gamma$) (A and B). Live cells recovered after 48 hours were assayed by ELISPOT as in Table 2.

FIGURE 7 shows the frequency of variant peptide antigen-induced IFN-$\gamma$ (Th1) (A) and IL-5 (Th2) (B) producing T cells as determined by ELISASPOT technique; (Represented as spot forming cells (SFC) in $10^6$ lymph node cells).

FIGURE 8 shows proliferation of T cell clones measured by [$^3$H]-thymidine incorporation after stimulation with varying concentrations of peptide variants.

FIGURE 9 shows a comparison of the proliferation pattern (A), and lymphokine, IL-4 (B) and IFN-$\gamma$ (C) secretion profile by a 9.4 Lys-specific T cell clone, 3C10, in response to 9.4 Lys (•), 9.4 Arg (▪) and 9.4 Met (▲).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IFN-$\gamma$ secreting T cell population or an IL-4 secreting T cell population. The method of the invention entails identifying peptides with low and high affinity binding for MHC and induction of T cell proliferation. Once identified, these T cell modulatory peptides are useful for treatment of subjects with Th1 or Th2 associated T cell disorders, such as allergic or autoimmune disorders.

In a first embodiment, the invention provides a method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IFN-$\gamma$ secreting T cell population, comprising preparing an array of variant immunodominant peptides from the antigen and selecting from the array, a peptide that has high MHC binding affinity and that induces proliferation of T cells specific for the antigen. As used herein, the term "T cell modulatory peptide" refers to a peptide that causes a change in a pre-existing T cell response. For example, the peptide can be used to divert a predominant T cell response away from a disease state by stimulating a protective T cell response. Th1 cells preferably secrete lymphokines such as IFN-$\gamma$, IL-2, and tumor necrosis factor (TNF). Therefore, when the Th2 response is dangerously high in a subject, such as in the case of allergic reactions, it is desirable to stimulate a Th1 response. Therefore, in contrast to preventing the deleterious response, the method of the invention operates by eliciting a protective response.

In another embodiment, the invention provides a method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IL-4 secreting T cell population, comprising preparing an array of variant immunodominant peptides from the antigen and selecting from the array, a peptide

that has low MHC binding affinity and that induces proliferation of T cells specific for the antigen. Th2 cells preferably secrete lymphokines such as IL-5, IL-6, IL-9, IL-10 and IL-13. Therefore, when the Th1 response is dangerously high in a subject, such as in the case of autoimmune disorders, it is desirable to stimulate a Th2 response.

The methods described above for identifying a T-cell modulatory peptide comprise the steps of preparing an array of variant immunodominant peptides from the antigen and selecting from the array a peptide that has either high or low MHC binding affinity, depending on the T cell population one desires to stimulate, and that induces proliferation of T cells specific for the antigen.

As used herein, "antigen" refers to a substance which elicits an immune response. The antigens of the invention which are sources for a T cell modulatory peptide may or may not be exogenously derived relative to the host. For example, the method of the invention may be used to identify a T cell modulatory peptide that is an "autoantigen". An autoantigen is a normal constituent of the body that reacts with an autoantibody or activates T cells. The invention also includes inducing tolerance to an "alloantigen". Alloantigen refers to an antigen found only in some members of a species, for example the blood group substances. Xenoantigens are substances which cause an immune reaction due to differences between different species.

An "immunodominant" peptide is a peptide derived from a whole antigen that is well-processed and presented to the T cell and is competitively favored among all of the potential determinants. The more poorly processed determinants from an antigen are termed "subdominant" and "cryptic" in descending hierarchical order of antigen presentation. In the method of the present invention, an immunodominant peptide is typically identified by immunizing an animal, such as a mouse, at a local site, for example intracutan-

eously in a footpad, removing the draining lymph nodes from the animal and preparing cell suspensions approximately 9-11 days post injection. The cells are distributed onto a solid support, such as the wells of a 96-well culture plate, to which, successively one of an overlapping set of peptides is added. Preferably, the overlapping peptides are approximately 10-20-mers, and most preferably 15-mers, with overlaps of from 10-14 amino acids. Antigen-induced T cell proliferation can be determined by standard methods known to those of skill in the art (See, Coligan, *et al.*, *Current Protocols in Immunology*, Wiley Interscience, 1991, Unit 3). Preferably, proliferation is assessed by measuring the incorporation of tritiated thymidine ([$^3$H]) to indicate DNA synthesis. In this way, one of skill in the art can define those peptides which induce a strong response as bearing a dominant determinant and those inducing a weak response as bearing a subdominant determinant. There will be several contiguous peptides that induce a response and by defining those amino acids found in each of the positive peptides, a "core" of residues may be identified.

Once an immunodominant peptide is identified, preferably the core of the determinant plus about 3 residues on each side of the core is used as the test immunogen. An array of variants at each amino acid residue within the core is produced, with about three variants at each residue. As used herein, a "variant" refers to a peptide which is a mutagenized form of an immunodominant or core peptide, or one produced through recombination or peptide synthesis, for example. Those of skill in the art will know standard methods for substitution of amino acid residues, such as any of the known methods for accomplishing random or site-directed mutagenesis of the DNA encoding the peptide. Typically, the three variants at each position of the wild-type sequence to be screened for the MHC binding affinity and induction of a T cell proliferative response are: (1) a conservative change; (2) a non-conservative change; and (3) a change to alanine variant. Where the wild-type residue is alanine, one of the residue changes will be to serine or a single small residue.

Amino acid changes which affect MHC binding can be evaluated directly or indirectly. For example, binding of a variant peptide can be determined directly by an assay using purified MHC molecules. Binding can be measured indirectly by a cellular competition assay. The peptides can be assayed for MHC binding in liquid phase or bound to a solid phase carrier. In addition, the peptides in these assays can be detectably labeled in various ways. Examples of types of assays which can utilize the peptides of the invention are competitive and non-competitive assays in either a direct or indirect format. Those of skill in the art will know, or can readily discern, other assay formats without undue experimentation.

The peptides of the invention can be bound to many different carriers and used to detect the binding of the peptide to MHC. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding peptides, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bio-luminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the peptides of the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the peptides of the invention can be done using standard techniques common to those of ordinary skill in the art.

According to the invention a high affinity peptide variant has an MHC binding affinity at least 1,000 times, preferably at least 5,000 times, greater than the corresponding wild-type peptide. Alternatively, a low affinity peptide variant of the invention has an MHC binding affinity at least 1,000 times, preferably at least 5,000 times, less than the corresponding wild-type peptide. Having established variants with high and low MHC binding affinities, T cells specific for the wild type peptide are stimulated with these variants and the lymphokine secretion pattern is determined. Amino acid changes which stimulate a T cell proliferative response can be assayed directly or indirectly. The method described above utilizing tritiated thymidine incorporation is an example of a direct assay. Induction of proliferation of the appropriate T cell population can be determined indirectly by measuring lymphokine production from a T cell population after stimulation with a variant immunodominat peptide. For example, induction of stimulation of proliferation of Th1 cells can be determined by assay for IFN-$\gamma$, IL-2, and/or tumor necrosis factor (TNF) and induction of stimulation of proliferation of Th2 cells can be determinined by assay for IL-5, IL-6, IL-9, IL-10 and IL-13. Preferably, the lymphokines are measured by standard immunoassay techniques, however, one of skill in the art will know of other assays for such detection. For confirmation of the T cell proliferative response, cloned T cells, as described below, are incubated with an increasing concentration of peptide variants in the presence of syngeneic antigen presenting cells (APCs). In the murine system, irradiated splenic cells are preferably used as APCs. Supernatants are then assayed, for example, by immunoassay or bioassay.

The method of the invention for identifying T cell modulatory peptides may further include the step of retesting the variants for their T cell specificity both *in vitro* and *in vivo*. For this purpose, T cell clones from primed lymph nodes are prepared which react with the wild type immunodominant peptide. These clones are prepared by methods known to those of skill in the art such as the

limiting dilution method described by Kimoto and Fathman (*J. Exp. Med.*, 152:759, 1980). These clones are then stimulated with each of the variant peptides *in vitro*. Animals, such as mice, are challenged with both the wild type peptide as well as the variant peptide and approximately 10 days later, lymph node proliferative responses are recalled with either peptide. Cross-reactivity in proliferative responsiveness, both in the *in vitro* and *in vivo* assays, indicates a similarity in T cell specificity observable with the response induced by variants and the wild type peptide.

The invention also provides a synthetic T cell modulatory peptide with the amino acid sequences ASQXRPSQR and YSDGSCTQRASEAHASLLPFN (SEQ ID NO:1 and SEQ ID NO:2, respectively), and conservative variations thereof, including the human analogs of these sequences. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. SEQ ID NO:1 represents amino acids 1 to 9 of myelin basic protein (MBP) polypeptide, wherein X is preferably glutamic acid, glutamine, alanine, valine, tyrosine or methionine. SEQ ID NO:2 represents amino acids 361 to 382 of promastigote surface protease, PSP (gp63) of *Leishmania major*. The core of SEQ ID NO:2 is amino acids RASEAHAS.

As used herein, the term "synthetic peptide" denotes a peptide which does not comprise an entire naturally occuring protein molecule. The peptide is "synthetic" in that it may be produced by human intervention using such techniques as chemical synthesis, recombinant genetic techniques, or fragmentation of whole antigen or the like.

The peptides of the invention include "functional fragments" of the peptides, as long as the activity of the peptide as noted above remains. Such peptides can be readily identified by those of skill using the routine screening methods described herein without resorting to undue experimentation. These peptides can be as few as 5, preferably as few as 10 amino acids in length.

Minor modifications of the primary amino acid sequence of the peptides of the invention may result in peptides which have substantially equivalent activity as compared to the specific peptides described herein or those identified by the method of the invention. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the peptides produced by these modifications are included herein as long as the biological activity of the original variant peptide still exists. For example, the first 6 amino acids of the peptide of SEQ ID NO:1 may stimulate Th1 cells to the same extent as the first 8 or 9 amino acids. Such determinations are routine and can be done without undue experimentation.

Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would also have utility. For example, one of skill in the art can use standard techniques to remove amino or carboxy terminal amino acids from SEQ ID NO:1, or delete amino acids from SEQ ID NO:2, as long as the amino acids are not required for biological activity of the particular peptide.

Peptides of the invention can be synthesized by such commonly used methods as t-BOC or FMOC protection of alpha-amino groups. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the C terminus of the peptide (See, Coligan, *et al.*, *Current Protocols in Immunology*, Wiley Interscience, 1991, Unit 9). Peptides of the invention can

also be synthesized by the well known solid phase peptide synthesis methods described by Merrifield, (*J. Am. Chem. Soc.*, 85:2149, 1962), and Stewart and Young, (*Solid Phase Peptides Synthesis*, Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

The peptides of the invention can be used singularly, in mixtures, or as multimers such as aggregates, polymers, and the like. Thus, the invention embraces synthetic peptides which comprise one or more of the same, or different, peptides of the invention to produce a homogeneous or heterogeneous polymer with respect to the particular peptides of the invention which are contained therein. Appropriate techniques for producing various mixtures, aggregates, multimers and the like will be known to those of skill in the art.

The invention also provides polynucleotides which encode the peptides of the invention. As used herein, "polynucleotide" refers to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. DNA encoding a peptide of the invention can be assembled from cDNA fragments or from oligonucleotides which provide a

synthetic gene which is capable of being expressed in a recombinant transcriptional unit. Polynucleotide sequences of the invention include DNA, RNA and cDNA sequences. A polynucleotide sequence can be deduced from the genetic code; however, the degeneracy of the code must be taken into account. Polynucleotides of the invention include sequences which are degenerate as a result of the genetic code.

The polynucleotide encoding the peptides of the invention includes a polynucleotide that encodes SEQ ID NO:1 or 2, as well as complementary nucleic acid sequences. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:1 or 2 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the peptides of the invention under physiological conditions.

Polynucleotide sequences encoding the peptides of the invention can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

The present invention also provides a method of modulating a T cell response to a specific antigen in a subject with a T cell disorder, comprising administering to the subject an effective amount of a T cell modulatory peptide for the antigen, wherein the peptide stimulates an IFN-$\gamma$ or an IL-4 secreting T cell population. When a subject has a T cell disorder associated with elevated

levels of Th2, the disorder is typically an allergic disorder or a helminth infection. When a subject has a T cell disorder associated with elevated levels of Th1, the disorder is typically an autoimmune disorder, or a disorder having a bacterial, viral or protozoan etiology. Preferably the subject is a human.

The term "effective amount" as used herein refers to the amount of T cell modulatory peptide administered is in sufficient quantity to decrease the subject's response to the antigen, for example, decrease the symptoms of an allergy. The amount of peptide that is considered effective can be determined by monitoring the antigen-specific Th1:Th2 ratio of a subject. Preferably, this is determined by ELISA measurements of lymphokines secreted by peripheral blood lymphocyte samples. Other methods will be known to those of skill in the art. The dosage ranges for the administration of the peptide of the invention are those large enough to produce the desired effect. Generally, the dosage will vary with the age, condition, sex, and extent of the infection with bacteria or other agent as described above, in the patient and can be determined by one skilled in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. In any event, the preadminstration and post administration ratio of Th1:Th2 should correlate with recovery of the patient.

Examples of allergic disorders include allergic rhinitis, asthma, atopic dermatitis, and food allergies. Examples of autoimmune disorders, where the immune system attacks the host's own tissues, include, but are not limited to, type 1 insulin-dependent diabetes mellitus, adult respiratory distress syndrome, inflammatory bowel disease, dermatitis, meningitis, thrombotic thrombocytopenic purpura, Sjögren's syndrome, encephalitis, uveitic, leukocyte adhesion deficiency, rheumatoid arthritis, rheumatic fever, Reiter's syndrome, psoriatic arthritis, progressive systemic sclerosis, primary biliary cirrhosis, pemphigus, pemphigoid, necrotizing vasculitis, myasthenia gravis, multiple sclerosis, lupus

erythematosus, polymyositis, sarcoidosis, granulomatosis, vasculitis, pernicious anemia, CNS inflammatory disorder, antigen-antibody complex mediated diseases, autoimmune haemolytic anemia, Hashimoto's thyroiditis, Graves disease, habitual spontaneous abortions, Reynard's syndrome, glomerulonephritis, dermatomyositis, chronic active hepatitis, celiac disease, autoimmune complications of AIDS, atrophic gastritis, ankylosing spondylitis and Addison's disease.

When desirable, T cells can be separated from other PBLs by techniques known in the art. Procedures for separation of cells may include magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, for example, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, for example, plate, or other convenient technique. Techniques providing accurate separation include fluorescence activated cell sorters, which can have varying degrees of sophistication, for example, a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

The T cell modulatory peptide is administered by any suitable means, including parenteral, subcutaneous, intrapulmonary, oral, and intranasal administration. Parenteral infusions include intramuscular, intravenous, intraarterial, or intraperitoneal administration. Preferably, the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or prolonged.

The peptides of the invention are administered in a physiologically acceptable solution. Preparations of peptide for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, and polyethylene glycol.

Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

A peptide of the invention can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. These include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acid, or organic acids such as acetic, oxalic, tartaric and the like. Salts also include those formed from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and organic bases such as isopropylamine, trimethylamine, histidine, procaine and the like.

The following examples are intended to illustrate, but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

## EXAMPLES

## EXAMPLE 1
## MATERIALS AND METHODS

1. Mice

Female BALB/cJ, C57BL/6, BALB.B, CBA/J, and 510.D2 mice (8-12 weeks old) were either purchased from Jackson Laboratories (Bar Harbor, ME) or bred in our own facilities.

2. Medium

HL-1 serum-free medium (Ventrex, Portland, ME) with 2 x $10^{-5}$M 2-mercaptoethanol, 20 mM glutamine, and 25 $\mu$g/ml gentamicin was used for pepscan experiments (FIGURES 1A and B and 2A and B). For other experiments, except where noted. RPMI 1640 (Sigma, St. Louis, MO) with 5% heat inactivated FCS (Gemini Bioproducts, Calabasas, CA), 2 x $10^{-5}$ M mercaptoethanol. 20 mM glutamine, and 25 $\mu$g/ml gentamicin was used.

3. Antigens

Recombinant gp63 (rPSP) was a generous gift of Dr. Robert McMaster (University of British Columbia); native gp63 (PSP) in either hydrophilic (HPSP) or amphiphilic (APSP) forms were kindly donated by Dr. Robert Etges (Universite de Lausanne). Soluble L. major (LV 39) 'antigen' was prepared by three cycles of freezing and thawing, followed by centrifugation at 10,000 g for 10 minutes.

4. Antibodies and lymphokines

XMG 1.2 (anti-mouse IFN-$\gamma$) as purified on a Protein G-Sepharose (Zymed, San Francisco, CA) column and biotinylated as described (Bayer, E.A., *et al.*, *Methods Biochem. Anal.*, 26:1, 1980); R46A2(ATCC) (anti-mouse IFN-$\gamma$) was

purified on a Protein G column as above; BVD4-1D11 and BVD6-2A62 (anti-mouse IL-4), TRFK-4 and -5 (anti-mouse IL-5), and JES5-sA5 and SXC-1 (anti-mouse IL-10) were purchased from Pharmingen (San Diego, CA). GK 1.5(anti-mouse $CD4^+$), 34-5-3S (anti $1\text{-}A^s$), and 2.43 (anti-mouse $CD8^+$) cells were purchased form ATCC and the antibodies purified in this laboratory. Anti-mouse $Thy\text{-}1^+$ ascites and rabbit Low-tox complement were purchased from Accurate Chemical and Scientific (Westbury, NY); rHuIL-2 was a gift of Dr. Ray Apple (Roche Molecular Systems, Alameda, CA); r-mouse IL-4 was a generous gift of Dr. William Paul (NIH), r-mouse IL-5 and IL-10 were purchased from Pharmingen; r-mouse IFN-$\gamma$ was purchased from Genzyme (Cambridge, MA).

5. Synthetic peptides

A complete series of overlapping peptides spanning the entire sequence of L major PSP (Miller, R.A., *et al.*, 39:267, 1990) were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia) using the 'multi-pin' peptide synthesis technique. The procedure has been modified as described in detail elsewhere also that the peptides can be cleaved from the pins (Maeji, N.J., *et al., J. Immunol. Methods*, 134:23, 1990). The first amino acid residue added in each case was proline followed by $\alpha$-Boc-$\varepsilon$-Fmoc-lysine. The Fmoc protecting group was then removed and additional Fmoc-protected amino acids were added onto the $\varepsilon$-amino group of the lysine by repetitive cycles of Fmoc deprotection and amino acid couplings. The terminal amino group of each peptide was acetylated. After removal of all the protecting groups, cleavage from the pins was performed by exposure to water at neutral pH, under which conditions the C-terminal lysine-proline residues formed a diketopiperazine derivative. Peptide yield was estimated as described (Maeji, N.J., *et al.*, *supra*).

6. Immunizations and culture conditions

Mice were immunized s.c. in a footpad with 2 μg of rPSP or 2.5 μg of either HPSP or APSP, or 40 μg of synthetic peptides. All immunogens were emulsified in 50% complete Freund's adjuvant (Difco, Detroit, MI). Mice were killed 9-10 days later and popliteal lymph nodes were pooled or prepared from individual mice and processed as single cell suspensions. Cells ($5 \times 10^5$/well) were seeded in 96-well plates (Costar, Cambridge, MA) along with various concentrations of peptide or protein, incubated for 3 days and then pulsed for 18 hours with 1 μCi of [$^3$H]thymidine (ICN, Costa Mesa, CA). Cultures were harvested with a Skatron cell harvester (Skatron, Sterline, VA) and [$^3$H]thymidine incorporation determined in a LKB-Wallac 1205 Beta-plate reader (LKB, Turku, Finland). For lymphokine production studies, lymph node cells were placed in 24-well plates (Costar) at 2-5 x $10^6$ cells/ml in 1 ml of medium along with antigen and incubated at 37°C. Supernatants were harvested after 48, 72 or 96 hours of culture, centrifuged, and stored at -70°C. For testing, T cell lines were incubated in 24-well plates at 1 x $10^5$ cells/ml along with 2 x $10^6$ mitomycin C-treated spleen cells and antigen, in 1 ml of medium. Supernatants were harvested after 24-48 hours and prepared as above. For ELISPOT assays, cells were incubated for 48 hours at $10^7$ cells/ml and viable cells recovered by flotation on Ficoll (d = 1.083), washed, and seeded in ELISPOT plates at various concentrations. Alternatively, lymph node cell suspensions were directly seeded in ELISPOT plates along with antigen and processed as described below.

7. Lymphokine measurements

IFN-γ, IL-4, and IL-10 were measured by ELISA as described (18-20). Briefly, 96-well ELISA plates (Nunc Maxisorp, Kamnstrup, Denmark) were coated with 2 μg/ml of capture antibody in 0.05 M Tris, pH 8.5, and incubated overnight at 4°C. The plates were then washed twice and blocked with 25 mM Tris-buffered saline - 100% FCS (blocking buffer) for 2 hours, washed four times,

and incubated with serial dilutions of samples in blocking buffer at room temperature for 90 minutes. After four washes, second antibodies were added at 2 μg/ml in blocking buffer, and the plates were then incubated for 1 hour at room temperatures, washed six times, and incubated with a 1:4000 dilution of avidin D-peroxidase (Vector, Burlingame, CA) for 30 minutes at room temperature. Finally, the plates were washed eight times and the substrate, 2.2'-azino-bis-3 ethylbenzthiazoline-6-sulfonic acid (ABTS: Sigma) added at 100 μg/ml along with $H_2O_2$ (1:2000 of a 30% solution). Optical density was measured using a Titertek Multiskan ELISA reader (Flow Laboratories, Huntsville, AL) at 405 nm. The values were converted to U/ml using recombinant protein as a standard.

To evaluate the apparent frequencies of Th1 and Th2 cells in the lymph nodes, an ELISPOT technique was used (Taguchi, T., *et al., J. Immunol. Methods*, 128:65, 1990; Fujihashi, K., *et al., J. Immunol. Methods*, 160:181, 1993). Briefly, Millititer HA plates (Millipore, Bedford, MA) were coated overnight at 4°C with 2 μg/ml of anti-IL-4, and IFN-γ, or anti-IL-5. Wells were then blocked by incubation with complete medium for 2 hours at room temperature. Cells were added to duplicate wells in concentrations ranging from $10^4$ to 2.5 x $10^5$ cells/well in 100 μl of medium and incubated for 20-60 hours at 37°C (see figure legends for details). Following incubation, plates were first washed extensively with ice-cold PBS with 0.01 M EDTA to remove adherent cells and then with PBS-0.05% Tween 20. The wells were then incubated with biotin-conjugated anti-IL-4 (2 μg/ml), anti-IFN-γ (1 μg/ml), or anti-IL-5 (2 μg/ml) for 90 minutes at room temperature, washed six times with PBS- Tween and reincubated with 1:2000 dilution of avidin D-peroxidase for 45 minutes at room temperature. Plates were then washed eight times and the spots developed by addition of 400 μg/ml of 3-amino-9-ethylcarbazole (AEC; Sigma) substrate

and $H_2O_2$ (1:1500 of a 30% solution) in 0.05 M sodium acetate buffer, pH 5.0, for 1 hours at room temperature. The number of spots was assessed with the aid of a dissection microscope.

8. Generation of T cell lines

Lymph node cells were prepared as described above and incubated in 24-well plates at a concentration of 5-10 x $10^6$ cells/ml in 1 ml of complete medium and antigen. Cells were harvested after 6 days and reincubated with fresh mitomycin C-treated syngeneic spleen cells [antigen presenting cells (APC)] and antigen. Seven days later, viable cells were recovered through flotation on Ficoll (Sigma) and incubated in 50 U/ml of rIL-2. From this point on, cells were stimulated with antigen/APC or IL-2 on alternate weeks.

## EXAMPLE 2

## DETERMINANT PROFILE OF T CELL PROLIFERATIVE RESPONSE TO PSP IN h-$2^d$ AND h-$2^b$ MICE

Using a series of 15mer peptides advancing along the molecule with 12 amino acid overlaps and covering the entire sequence of native L. major PSP, as derived from the DNA sequence of the cloned gene (Miller, R.A., *et al.*, *Mol. Biochem. Parasitol.* 39:267, 1990), the general profile of the T cell proliferative response to HPSP or rPSP was determined. FIGURE 1 shows profiles of the determinant regions on the PSP molecule in BALB/c and B6 mice. Lymph node cells of four to six pooled BALB/c (A and B) or B6 (C and D) mice previously immunized with 2 $\mu$g of rPSP (A and C) or 2.5 $\mu$g of HPSP (B and D) were cultured in 28 $\mu$M solutions of each of 155 peptides for 72 hours and then pulsed for an additional 18 hours with 1 $\mu$Ci of [$^3$H]thymidine. Peptides were 15mers advancing by three residues and cover the entire PSP molecule. Peptide number refers to the N-terminal residue of the 15mer. Data are

expressed as [$^3$H]thymidine incorporation in individual cultures in FIGURES 1, 2 and 3. To assure reproducibility, experiments were performed two to four times.

Several T cell determinant regions are readily apparent in BALB/c mice after HPSP immunization (FIGURE 1A). The most dominant determinant region was one that can be recalled with peptides p361 ('p361' is the 15mer starting at residue 361) through p367 followed by a group of subdominant determinants recalled by peptides (in order to importance) p403 through p409, p136 through p151, p106 through p121, p172 through p181, and p286 through p292 respectively, as well as several other minor regions. The determinants are referred to by a single number representing the amino terminus of the 15mer peptide giving the peak response.

The only difference noted between recombinant (FIGURE 1B) and native PSP immunization was the loss of a determinant in the recombinant profile in the region around residue 331. In B6 mice (FIGURE 1C and D), a similar number of dominant and subdominant determinants were found although the total number of determinant regions is smaller than in BALB/c mice. The most predominant determinant is recalled by peptides p451 through p460, while other determinants are within peptides p187 through p193, p136 through p148, and p106 through p112. Interestingly, ≈50% of these determinants are also present in BALB/c mice, although others are clearly different.

## EXAMPLE 3

## CORE DETERMINANTS IN THE RESPONSE TO PSP

In a second set of experiments, synthetic peptides, overlapping except for a single amino acid, corresponding to major determinant regions detected in the previous experiments, were used to recall responses.

FIGURE 2 shows fine determinant mapping of PSP determinant regions in BALB/c mice. Lymph node cells of individual BALB/c mice previously immunized with 2.5 $\mu$g of HPSP were cultured along with each peptide (at 14 $\mu$M) within determinant regions with cores 138-147/143-155 (A) and 369-376 (B) under the same conditions used in FIGURE 1. Peptides are 15mers advancing by one residue and cover selected determinate regions represented in FIGURE 1.

FIGURE 3 shows fine determinant mapping of PSP determinant regions in B6 mice. Lymph node cells of individual B6 previously immunized with 2.5 $\mu$g of HPSP were cultured along with each peptide (at 14 $\mu$M) within determinant regions with cores 138-147/143-155(A), 194-201(B), or 456-464 (C) under the same conditions used in FIGURE 1. Peptides are 15mers. advancing by one residue and cover selected determinant regions shown in FIGURE 1.

FIGURE 4 shows core sequences for determinant regions defined in FIGURE 2. The cores are defined according to the proliferation results form FIGURE 2 and include shared residues of peptides which induce at least 50% of the maximal proliferation for that determinant region. PSP peptide sequences are indicated by the single-letter amino acid code.

Recall by the 15mer overlapping peptide series (pepscan) after HPSP immunization allowed the core regions, which are here arbitrarily defined as those residues contained in all peptides giving 50% or more of the maximal proliferation for the determinant region, of each determinant to be mapped. FIGURE 2(A and B) shows the pepscan for the regions surrounding peptides p136 through p151 and p361 through p367 respectively in BALB/c mice, and FIGURE 3(a-c) shows regions that include peptides p136 through p148, p187 through p193, and p451 through p460 respectively in the B6 mice. Core regions for each determinant are illustrated in FIGURE 4. It is noteworthy that determinant regions 136-151 and 361-367 gave clearly similar cores for BALB/c and B6 mice. The determinant region 136-151 clearly contains two overlapping determinants in both BALB/c and B6. In BALB/c the cores are 138-148 and 144-155, and in the B6, 138-147 and 143-155. The determinant region p361-p367 gave exactly the same core, 369-376, for BALB/c and B6 strains. Experiments using BALB.B (H-$2^b$) and B10.D2 (H-$2^d$) gave similar responses to B6 and BALB/c respectively.

It is interesting that some determinants, such as the BALB/c one with the core 369-376 show very active proliferation to peptides which start in nine contiguous positions, suggesting the existence of multiple overlapping cores. Quite large determinant regions are also present in B6 mice, e.g., in regions with the cores 456-464 and 194-201.

## EXAMPLE 4

## LYMPHOKINE PROFILE INDUCED BY DOMINANT DETERMINANTS

Several studies have shown (Sadick, M.D., *et al., J. Exp. Med.*, 171:115, 1990; Sadick, M.D., *et al., J. Immunol.*, 136:655, 1986; Belosevic, M., *et al., J. Immunol.*, 143:266, 1989) importance of IFN-$\gamma$, as well as the deleterious role of IL-4 in the resolution of murine leishmaniasis. A candidate vaccine peptide in Leishmania systems should induce consistently high IFN-$\gamma$ production and low IL-4 levels. Lymphokine measurements after peptide recall within the major determinant regions in BALB/c and B6 are shown in FIGURE 5. FIGURE 5 shows lymphokine profile for the major PSP determinant regions in BALB/c and B6. Lymph node cells ($5 \times 10^6$) from individual BALB/c (A. mice 1 and 2) and B6 mice (B mice 1 and 2) were cultured with peptides corresponding to determinant regions with cores 369-376 (BALB/c) and 456-464 (B6) in 1 ml of complete medium. Supernatants were collected after 48 hours centrifuged, stored at -70°C and thawed 10 minutes before assay for IFN-$\gamma$ and IL-4 by ELISA cultures were established as in FIGURE 2 and assayed for proliferation. The results express the lymphokine and proliferation profiles from two individual animals for both BALB/c and B6.

There is a close correlation between proliferation and IFN-$\gamma$ production in these determinant regions in each strain, with nearly exact agreement among individuals. No IL-4 was detected for these dominant determinants when measured by ELISA. T cell lines developed form these dominant regions also show strong IFN-$\gamma$ production. Nevertheless, with T cell lines, some peptides related to the B6 determinant region with core 456-464 were also able to induce IL-4 secretion.

## EXAMPLE 5

## PEPTIDE PRIMING FOR LEISHMANIA RECALL

Combining the results of proliferation and IFN-γ production dominant peptide 364-378 in BALB/c mice and peptide 452-464 in B6 mice were used to determine whether, after peptide priming, specific T cell activity could be recalled by a frozen and thawed Leishmania extract. Lymph node cells from either immunized BALB/c or B6 mice were able to proliferate in response to the *L. major* extract and produced IFN-γ but no measurable IL-4 (Table 1). Although a proliferative response with heat-denatured HPSP (HPSP) could not be recalled, the IFN-γ response was positive. However, several T cell lines against HPSP were developed from both BALB/c and B6. Also, recall with rPSP was easily obtainable.

### TABLE 1

### IMMUNIZATION WITH PSP PEPTIDES LEAD TO A Th1 RECALL RESPONSE WITH *L. major* ANTIGEN

| Recall Antigens | BALB/c | | | B6 | | |
|---|---|---|---|---|---|---|
| | Δc.p.m. | IFN-γ (U/ml) | IL-4 (U/ml) | Δc.p.m | IFN-γ (U/ml) | IL-4 (U/ml) |
| Heat denatured HPSP | 2989 | 38.8 | <3 | 2961 | 138.8 | <3 |
| *L. major* extract | 35.086 | 236.6 | <3 | 45.986 | 122.6 | <3 |
| Medium | -- | <0.8 | <3 | --- | <0.8 | <3 |

BALB/c or B6 mice were immunized with 40 μg of peptide 364-378 or 452-466 respectively. Nine days later popliteal lymph node cells were pooled form three animals and assayed for proliferation and lymphokine production in response to a frozen and thawed extract of *L. major* ($1 \times 10^6$/ml). Proliferation was measured as described in Example 2 and lymphokine production as in Example 5 (c.p.m. values for medium alone were $4\text{-}6 \times 10^{-3}$).

## EXAMPLE 6

## LYMPHOKINE INDUCTION BY DIFFERENT PEPTIDES WITHIN THE DETERMINANT ENVELOPE

ELISPOT assays were performed to determine the apparent frequencies of IFN/IL-5 producing T cells in lymph nodes from BALB/c, B6 and CBA mice immunized with peptide 364-378 (Table 2). The frequency of IFN-$\gamma$ producing cells was =50-100 times that of IL-5 producing cells but cells producing IL-5 were clearly measurable. Most interesting were results obtained form ELISPOT assays performed with lymph node cells from mice immunized with whole rPSP (Table 3). Different sequences within a determinant envelope gave rise to T cells with different patterns of lymphokine production. For example, a central peptide such as 364-378 almost exclusively stimulated a population of cells with a typical Th1 phenotype while peptide 368-382 induced a frequency of IL-4 producing cells that was up to three times higher than that of IFN-$\gamma$ producing cells; a similar pattern was induced by the other boundary peptide, 361-375.

## TABLE 2

## IMMUNIZATION WITH PEPTIDE 364-378 TO A STRONG PREDOMINANTLY Th1 RESPONSE IN THREE STRAINS OF MICE

No. of spot forming cells per $10^6$ cells

| Recall antigen | BALB/c IFN | IL-5 | B6 IFN | IL-5 | CBA IFN | IL-5 |
|---|---|---|---|---|---|---|
| P364-378 | 1500 | 20 | 1900 | 24 | 4800 | 32 |
| rPSP | 2000 | 35 | 1800 | 37 | 4700 | 34 |

Balb/c, B6 or CBA mice were immunized with p364-378 and pooled popliteal lymph node cells from three animals were assayed for their ability to secrete lymphokines. Cells were cultivated at $10^7$ cells/ml for 48 hours with either 10 $\mu$g/ml of p364-378 or 3 $\mu$g/ml of rPSP and then dispersed, washed, and seeded in ELISPOT plates at concentrations from $10^4$ to 2.5 x $10^5$ cells/well in duplicate. The plates were cultivated for an additional 20 hours and then processed for spot development as described.

## TABLE 3

## APPARENT RATIO OF Th1/Th2 INDUCTION IS INFLUENCED BY RECALL PEPTIDE

Ratio of IFN-γ/IL-4 producing cells

| Recall peptide | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| p360-374 | n.d. | (240/440) | 0.76 | (100/130)[a] |
| p361-375 | 0.55 | | 0.54 | ( 60/100) |
| p362-376 | n.d. | | 1.30 | (120/90) |
| p363-377 | n.d. | | 2.50 | (100/40) |
| p364-378 | 16.25 | (1300/90) | 10.80 | (650/60) |
| p365-379 | n.d. | | 2.00 | (400/200) |
| p366-380 | n.d. | | 5.80 | (350/60) |
| p367-381 | n.d. | | 2.36 | (260/110) |
| p368-382 | 0.38 | (320/840) | 0.61 | (130/210) |
| p369-383 | n.d. | | 1.25 | (100/80) |
| p370-384 | n.d. | | 1.10 | ( 90/80) |

[a] Number of IFN-γ/IL-4 producing cells per $10^6$ cells.

Different T cells with the same general specificity for this determinant region, but with different fine specificity requirements may be present and differentially stimulated by these peptides. Since it has been suggested that development of Th1 and Th2 cells are mutually inhibitory events (Hsieh, C.S., *et al.*, *Science*, 260:547, 1993), and that the initial concentration of Th1/Th2 lymphokines is critical, it is possible that the total dominance of IFN-γ in most of the determinant regions measured is a result of the inability of those peptides to prime a sufficient number of Th2 cells or to induce secretion of enough IL-4 by primary T cells to permit the development of Th2 cells.

To test this possibility, BALB/c mice were immunized with rPSP and the ability of lymph node cells to produce IL-5 when anti IFN-γ antibodies were added to the cultures together with peptides 364-378 or 368-382 was tested. FIGURE 6 shows peptides within the determinant region with core 369-376 are able to

induce Th2 cells in the presence of anti-IFN-$\gamma$. BALB/c mice were immunized with 2.5 $\mu$g of HPSP (A and B) and injected i.p. with 500 $\mu$g of XMG 1.2 antibody in PBS (B only) on days 1 and 3. On day 9, popliteal lymph node cells from three animals were pooled from each group and cultivated for 48 hours with peptides 364-378 (A and B) or 368-382 (A) in the presence or absence of 20 $\mu$g/ml of antibody R46A2 (anti-IFN-$\gamma$) (A and B). Live cells recovered after 48 hours were assayed by ELISPOT as in Table 2.

ELISPOT results present in FIGURE 6(A) show that the apparent dominance of IFN-$\gamma$ producing cells in response to peptide 364-378 can be altered markedly with a large increase in IL-5-producing cells. This suggests that peptide 364-378 induces not only IFN-$\gamma$ from the former population. A similar increase in Th2 was observed *in vivo* when mice were injected with anti-IFN-$\gamma$ (FIGURE 6B). As can be seen in FIGURE 6(A) when peptide 368-382 was used for recall, there was a clear decrease in the number of IFN-$\gamma$ secreting T cells relative to the number of IL-5 secreting T cells. The effect is not as great as that in Table 3 which may be attributable to a number of differences in the two experiments including a difference in immunogens (rPSP versus HPSP) and a difference in the methodology for measure single T cells.

## EXAMPLE 7

## VARIANT PEPTIDE MHC BINDING

An *in vivo* (polyclonal) as well as an *in vitro* (monoclonal) system was used to determine whether the same T cell displays a distinctive lymphokine secretion pattern when stimulated with differential MHC-binding ligands under identical stimulatory conditions, for example, APCs expressing identical MHC molecules ($I\text{-}A^u$), costimulatory molecules and cytokines. The N-terminal peptide fragment of myelin basic protein, acetylated 1-9 (abbreviated here as 9.4Lys), served as

the desired ligand because single amino acid substitutions at position 4 allowed the generation of variants with an enormous range of MHC-binding affinities coupled with unchanged T cell specificity (Wraith, D.C., *et al., Cell*, 59:247-255, 1989; Hood, L.E., *et al., Cold Spring Harbor Symp. Quant. Biol.*, 54:859-874, 1989). Thus, (i) 9.4Lys-specific T cell clones derived from B10.PL mice proliferated to all position 4 variants tested; (ii) the pattern of polyclonal recall with variants after challenging B10.PL mice with 9.4Lys or its variants remain identical (Kumar, *et al.*, in preparation), (iii) cloned T cells specific for the peptide variants (9.4Ala, 9.4Met and 9.4Val), derived after challenging B10.PL mice with the respective peptides, recognized each of the variants with a profile identical to that of 9.4Lys-specific T cells derived after 9.4Lys immunization).

In order to choose ligands with the highest and lowest affinities, the relative MHC-binding affinities of the variants using the purified I-$A^u$ molecule and a prototype labelled peptide were determined. These are expressed as 50% $\mu$M doses of the competing peptides as shown in Table 4. MHC-binding affinities were determined using a direct MHC-binding assay using purified class II molecules. Purified I-$A^u$ (10 nM to 1 mM was incubated with 5 nM of the radiolabeled non-natural peptide YAHAAHAAHAAHAAHAA (SEQ ID NO:3) for 48 hours in PBS containing 5% DMSO in the presence of a protease inhibitor mixture (1mM PMSF, 1.3 mM 1.10 phenanthroline, 73 $\mu$M pepstatin A, 8 mM EDTA, 6 mM N-ethylmaleimide, and 200 $\mu$M N-p-tosyl-L-lysine chloromethyl ketone) according to the standard protocol (Buus, S., *et al., Science*, 235:1353-1358, 1987). The final detergent concentration in the incubation mixture was 2.6% digitonin. The amount of MHC required for 10-20% binding was approximately 20-100 nM. All subsequent assays were then performed using this class II concentration. In inhibition assays, peptide competitors were

typically tested at concentrations ranging from 1.2 mg/ml to 0.12 ng/ml. The data were then plotted and the dose yielding 50% inhibition was calculated. Each peptide was tested in 2-4 completely independent experiments.

Peptides were synthesized by Drs. S. Horvath (Caltech, Pasadena, CA) or C. Miles (Macromolecular Resources, Fort Collins, CO) using a solid phase technique on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA) and were purified on a reversed phase column by HPLC (Clark-Lewis, I., *et al.*, *Science*, 231:134, 1986). I-$A^u$ molecules were purified from a I-$A^u$ expressing hybrid cell line JK 91.7 that was produced by fusion of the BALB/c B cell lymphoma line A20.2 JA (I-$A^d$, I-$E^d$) to spleen cells of a PL/J mouse (I-$A^u$, I-$E^u$). Cells were lysed at a concentration of $10^8$ cells/ml in PBS/1% NP40, and 1 mM PMSF. The lysates were cleared of nuclei and debris by centrifugation at 15,000 g and the I-$A^u$ molecule was affinity purified as previously described (Wall, M. *et al.*, *Intl. Immunol.*, 4:773-777, 1992). To avoid contamination with $A^d$ and mixed isotype molecules, Mab Y3JP, which recognizes I-$A^u$ but not I-$A^d$ or mixed isotypes, was used.

Variant peptides with higher T cell stimulatory capacities bind more effectively than 9.4Lys as suggested earlier (Wraith, D.C., *et al.*, *supra*; Hood, L.E., *et al.*, *supra)*. Peptide variant 9.4Arg is the poorest binder among all variants whereas peptides 9.4Met and 9.4Tyr, which bind almost 10,000 times better than 9.4Lys, are the best binders. In particular, the wild type peptide 9.4Lys is only slightly better than 9.4Arg. Peptide variant 9.4Ala showed a 400 fold better binding affinity than 9.4Lys, consistent with earlier observations (Wraith, D.C., *et al.*, *supra*; Hood, L.E., *et al.*, *supra*). Interestingly, the longer N-terminal peptide of MBP, Ac1-20, binds almost as effectively as 9.4Ala with I-$A^u$. These findings clearly indicate that the residue at position 4 in 9.4Lys is involved in MHC binding.

**TABLE 4**

**RELATIVE BINDING OF 9.4 LYS AND ITS POSITION 4 VARIANTS TO I-A$^{u}$**

| PEPTIDES | AMINO ACID SEQUENCE | 50% μM DOSE | RELATIVE BINDING RATIO TO 9.4 LYS |
|---|---|---|---|
| 9.4Arg | Ac-ASQRRPSQR | 26 | <0 or -3 |
| 9.4Lys | Ac-ASQKRPSQR | 7.4 | -- |
| 9.4Glu | Ac-ASQERPSQR | 2.6 | 3 |
| 9.4Gln | Ac-ASQQRPSQR | 0.32 | 25 |
| 9.4Ala | Ac-ASQARPSQR | 0.019 | 400 |
| 9.4Val | Ac-ASQVRPSQR | 0.0020 | 4000 |
| 9.4Tyr | Ac-ASQYRPSQR | 0.00069 | 10,000 |
| 9.4Met | Ac-ASQMRPSQR | 0.00064 | 10,000 |

Ligands with high or low MHC-binding capabilities were tested to determine whether they could result in tilting the response towards Th1 or Th2. 9.4Arg and 9.4Lys were used to represent the poorest, and 9.4Met the best MHC-binders in our stimulation assays. B10.PL mice were immunized with 9.4Arg, 9.4Lys or 9.4Met and 10 days later, frequencies of Th1 or Th2 cells were assessed in draining lymph nodes using cytokine-specific, single cell, enzyme-linked immunospot (ELISA SPOT) (Taguchi, T., *et al., J. Immunol. Methods,* 128:65-73, 1990; Fujihashi, K., *et al., J. Immunol. Methods,* 160:181-189, 1993) assays (FIGURE 7) and represented as spot forming cells (SFC) among $10^6$ lymph node cells. The very sensitive ELISA single cell spot assay was used, which measures the frequency of cells actually secreting a given lymphokine and eliminates problems associated with adsorption or degradation of

lymphokines in ELISA as well as difficulties in quantitation of translated or secreted product in mRNA detection. (At the polyclonal level in lymph node cells, IL-4 production was undetectable by ELISA, owing to either low levels or cross-regulation by IFN-$\gamma$)

Groups of B10.PL mice (4 in each group) were primed subcutaneously with 7 n moles of peptide emulsified in complete Freund's adjuvant. Nine days later, draining popliteal and inguinal lymph node cells ($1 \times 10^7$) were activated *ex-vivo* with an optimum concentration of peptides (9.4Lys, 14 $\mu$M; 9.4Arg, 50 $\mu$M; 9.4Met, 7 $\mu$M) in 1 ml of complete medium (DMEM). Lymph node proliferative recall responses were similar at these concentrations. After a 48 hour culture, live cells were recovered by passage through Ficoll-Hypaque (d=1.083), washed and transferred by serial dilution (from $10^4$ to $5 \times 10^5$ cells/well) to 96-well microtiter plates (Millipore) that had been precoated with mAb R4-6A2 (anti-IFN-$\kappa$) and TRFK-5 (anti-IL-5) (Pharmingen, San Diego, CA). After 24 hours, cells were removed and IFN-$\kappa$ and IL-5 spots were visualized using biotinylated XMG1.2 and TRFK-4 and avidin D-peroxidase in conjunction with 3-amino-9-ethylcarbazole (SIGMA) substrate (SIGMA). Spots were counted under a dissecting microscope and the frequency of antigen-specific cells was determined from the difference between the number of spots seen with and without antigen. In two other independent experiments, the frequencies of IFN-$\kappa$ producing cells in mice challenged with 9.4Met were 4.1 or 5 fold higher than in mice immunized with 9.4Lys/9.4Arg. In contrast, frequencies of IL-5-producing cells were comparable in each group of mice.

Clearly, IFN-$\gamma$ producing cells were about 5 times higher in mice challenged with the high affinity peptide, 9.4Met (1170 cells per $10^6$ cells) in comparison with mice immunized with the low affinity peptide, 9.4Arg or 9.4Lys (250 cells per $10^6$ cells) (FIGURE 7A). In contrast, the frequency of IL-5 producing cells was comparable in all three groups of mice (70-80 cells per $10^6$ cells) and did

not increase proportionately in response to the highest affinity ligand (FIGURE 7B). Similarly, the frequency of IL-4 secreting cells was comparable in each group. The ratio of the frequencies of IFN-γ/IL-5 producing T cells was 15 in 9.4Met-immunized mice in comparison to 2 or 3 in 9.4Arg or 9.4Lys challenged mice. Thus, the high affinity ligand appeared to shift the lymphokine-secretion pattern more towards the Th1-type. To attempt to compensate for the poor binding capacity of 9.4Arg, mice were challenged with a 50-100 fold higher concentration of 9.4Arg. This resulted in a 10-fold increase in the number of IL-5-secreting cells without a proportionate increase in the frequency of IFN-γ producing T cells (the IFN-γ/IL-5 ratio being less than 1).

## EXAMPLE 8

## STIMULATION OF T CELLS WITH PEPTIDE VARIANTS

To determine whether the same T cell could produce IFN-γ or IL-4 upon differential stimulation with altered ligands, cloned T cells specific for 9.4Lys were exposed to a concentration range (from .001 nM to 1 mM) of each of the peptides, and then their proliferation and lymphokine secretion pattern was measured.

First, 9.4Lys-specific T cell clones (Bhardwaj, V., *et al.*, *J. Immunol.*, 151:5000-5011, 1993; Urban, J., *et al.*, *Cell*, 54:577-592, 1988) were studied to determine whether they were capable of recognizing variants with low as well as high MHC-binding affinities. A typical proliferation profile of a $CD4^+$ T cell clone (3C10) specific for 9.4Lys, which secreted interleukin 4 (IL-4) but not interferon-γ, is shown in FIGURE 8. Briefly, T cells ($5 \times 10^4$-$1 \times 10^5$ cells per well) (T cell clone, 3C10, was derived from a long term T cell line, generated from B10.PL mice immunized with MBP, by two sequential limiting dilutions at 0.3 cells per well) were incubated with irradiated syngeneic spleen cells (APC) ($1 \times 10^5$-$5 \times 10^5$ cells per well) in the absence or presence of peptides at different

concentrations (1nM to 14 μM). Proliferation of T cell clones was measured by [$^3$H]-thymidine incorporation for the last 18 hours of a 3-day culture. Two days later, 1 μCi of $^3$H-thymidine was added to each well. Plates were harvested 18 hours later and proliferation was determined by scintillation counting as before (Kumar, V. *et al.*, *J. Exp. Med.*, 178:909-916, 1993). Amino acid sequences of peptides are shown in Table 1. Ac1-9 (•), 9.4Glu (○), 9.4Gln (△), 9.4Ala (▽), 9.4Arg (▪), 9.4Met (▲), and 9.4Val (▫). These data are representative of two separate experiments.

Peptide variants with position 4 residues possessing hydrophobic side chains smaller than lysine or a negative charge (Glu) did not significantly affect stimulation, whereas amino acid substitutions with either neutral or uncharged polar side chains (gln) were more effective in T cell activation. Notably, position 4 variants of 9.4Lys with nonpolar or hydrophobic side chains (ala, val, met) showed a strong heteroclitic proliferative response in that they were able to efficiently stimulate T cells at a $10^3$ to $10^5$-fold lower antigen concentration (FIGURE 8). The proliferative hierarchy of these variants was not unique to this T cell clone, as other 9.4Lys-specific cloned T cells (2C6, 5C4 and PL2.2) displayed a similar profile of stimulation (Bhardwaj, V., *et al.*, *J. Immunol.*, 151:5000-5011, 1993)

## EXAMPLE 9

## PROLIFERATION AND LYMPHOKINE SECRETION IN RESPONSE TO PEPTIDE VARIANTS

The relative capability of variants to induce proliferation and IL-4-secretion was compared (FIGURE 9). A comparison of the proliferation pattern (A) and lymphokine, IL-4 (B) and IFN-$\gamma$ (C) secretion profile by a 9.4Lys-specific T cell clone, 3C10, in response to 9.4Lys (•), 9.4 Arg (▪) and 9.4Met (▲) was performed.

$5 \times 10^4$ cloned T cells/well were incubated with $5 \times 10^5$ syngeneic APC (3000R-irradiated splenic cells from B10.PL mice) in the presence of varying concentrations (from .001 nM to 1.0 mM) of peptides, 9.4Lys, 9.4 Arg and 9.4Met in 96-well microtiter plates in triplicates. Proliferation was measured by ($^3$H)-thymidine incorporation as in Example 8. For the measurement of IFN$\gamma$ and IL-4, plates were incubated at 37°C and supernatants were harvested after 48 and 72 hours of culture, centrifuged and stored at -70°C. IFN-$\gamma$ and IL-4 were measured by ELISA as described (Curry, R.C., *et al., J. Immunol. Methods,* 104:137-145, 1987; Mosmann, T., *et al., J. Immunol.* 145:2938-2945, 1990). Briefly, 96-well ELISA plates (NUNC Maxisorp, Kamnstrup, Denmark) were coated with 2 $\mu$g/ml of capture antibody in 0.05 M Tris, pH 8.5, and incubated overnight at 4°C. The plates were then washed 2 times and blocked with 25 mM tris-buffered saline-10% FCS (blocking buffer) for 2 hours, washed 4 times and incubated with serial dilutions of samples in blocking buffer at room temperature for 90 minutes. After 4 washes, second antibodies were added at 2 $\mu$g/ml in blocking buffer, the plates were incubated for 1 hour at room temperature, washed 6 times, and then re-incubated with a 1:4000 dilution of avidin D-peroxidase (Vector, Burlingame, CA) for 30 minutes at room temperature. Finally, the plates were washed 8 times and the substrate, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (SIGMA, St. Louis, MO)

added at 100 μg/ml along with $H_2O_2$ (1:2,000 of a 30% solution). O.D. was measured using a Titertek Multiskan ELISA reader (Flow Laboratories, Huntsville, AL) at 405 nm. The values were converted to units/ml using recombinant protein as a standard. The lower limits of detection were 0.8 and 0.5 units/ml for IFN-γ and IL-4, respectively. Murine IFN-γ-specific monoclonal antibodies R4-6A2 and XMG 1.2 and anti-mouse IL-4 monoclonals, BVD4-1D11 and BVD6-2A62 were purchased from Pharmingen, San Diego, CA. Recombinant mouse IL-4 was a generous gift of Dr. William Paul (NIH) and r-IFN-γ was purchased from Genzyme Co., Cambridge, MA. These data are representative of three independent experiments.

Clearly, all three peptides, 9.4Lys, 9.4Arg and 9.4Met were able to induce proliferation and IL-4 production, as expected of an established Th2 clone (FIGURE 9A and 9B). Interestingly, the concentration required for 50% maximal IL-4 production upon stimulation with 9.4Met was only about 10-fold lower (0.3 μM) than that required for 9.4Arg/9.4Lys (4 or 7 μM), while in a similar comparison of "proliferation efficiency", 9.4Met is about $1\text{-}5x10^5$ times better than 9.4Lys. In contrast, 9.4Arg is 10-fold poorer in inducing proliferation, while the concentration required for 50% maximal IL-4 production appears to be similar to that of 9.4Lys. These data suggest that variants with higher MHC-binding affinity are relatively better in inducing proliferation than IL-4 production and conversely, variants with poor MHC-binding induce IL-4 secretion more effectively.

Importantly, 9.4Met induced significant levels of IFN-γ secretion in 2/4 T cell clones although at a relatively high peptide concentration (FIGURE 9C). The maximum amount of IFN-γ produced by the T cell clone 3C10 was 47.1 u/ml at 100 μM of 9.4Met. Similarly, other variants which demonstrated more than 1000-fold higher MHC-binding than 9.4Lys, for example, 9.4Val, also induced IFN-γ. The 9.4Lys and 9.4Arg peptides with very low MHC-binding were not

able to induce $\gamma$-IFN, even at very high peptide concentrations (up to 1 mM). To ensure that IFN-$\gamma$ was secreted by T cells but not by other cell-types, for example, APCs, in the presence of 9.4Met in the ELISA assays, purified T cells (3C10) were used to measure IFN-$\gamma$ production by the ELISA spot technique. The frequency of IFN-$\gamma$ and IL-4-producing cells after stimulation with 9.4Met (28 $\mu$M) was 1/800 and 1/80 T cells, respectively.

## EXAMPLE 10

## IL-4 PRODUCTION IN T CELLS AFTER LOW AFFINITY PEPTIDE TREATMENT

Cytokine production by clone 3C10 at similar levels of the different variants on the surface of the antigen-presenting cell was determined. In the absence of any direct measure of surface antigen/MHC display, this was assessed indirectly, using the 9.4Lys-reactive T cell hybridoma (HY.33). Peptide concentrations of the variants were determined for which they have apparently triggered similar numbers of TCR as measured by proliferation of an indicator T cell line (Table 5). The 9.4Lys-specific T cell hybridoma (Hy.33) cells ($1x10^5$ cells/well), were stimulated in the presence of syngeneic irradiated spleen cells ($5x10^5$) and various concentrations of the peptides. After 24 hours, IL-2 secretion was determined by measuring $^3$H-thymidine uptake by the IL-2-dependent cell line, HT-2, in the presence of 100 $\mu$l of 24 hours culture supernatant as before (Bhardwaj, V., *et al.*, *J. Immunol.*, 151:5000-5001, 1993). The control (APC and T cells without antigen) CPM were 1488+ 138. The data shown are the arithmetic means of triplicate wells. Cytokine production by T cell clone 3C10 was measured by ELISA as in Example 9. Proliferation as well as lymphokine production is compared at a linear range for each of the peptides.

As expected, the concentrations required for the same level of TCR-signalling were different for different variants, for example, 14 $\mu$M, 0.7 $\mu$M and 0.0007 $\mu$M for 9.4Arg, 9.4Lys and 9.4Met, respectively. At these peptide concentrations, the T cell clone, 3C10, did not produce detectable levels of IFN-$\gamma$ (Table 5). Interestingly, a significant amount of IL-4 was produced in the presence of 9.4Arg or 9.4Lys. This further confirms that lower affinity variants preferentially trigger IL-4 production.

**TABLE 5**

**LIGANDS WITH LOWER MHC BINDING PREFERENTIALLY INDUCE IL-4 SECRETION BY T CELLS**

| PEPTIDES | Signalling in T cell hybridoma, Hy.33 | | Lymphokine production by T cell clone, 3C10 (units/ml) | |
|---|---|---|---|---|
| | Peptide concentrations ($\mu$M) | Proliferation (CPM) | IFN-$\gamma$ | IL-4 |
| 9.4Arg | 14 | 8266 $\pm$ 583 | <0.8 | 220 $\pm$ 18 |
| 9.4Lys | 0.7 | 8977 $\pm$ 655 | <0.8 | 31 $\pm$ 10 |
| 9.4Met | 0.0007 | 8187 $\pm$ 400 | <0.8 | <3 |

These data suggest that changes in MHC-binding affinity result in altered T cell signaling leading to a differential lymphokine secretion pattern. This is consistent with recent demonstrations of altered signaling in T cells as a result of recognition of agonist/antagonist peptides (Evavold, B.D., *et al.*, *Science.*, 252:1308-1310, 1991; de Magistris, *et al.*, *Cell*, 68:625-634, 1992). It has been suggested that high affinity peptides can form a dense array of ligands (Murray, J.S., *et al.*, *J. Immunol.*, 22:559-565, 1992; Soloway, P., *et al.*, *J. Exp. Med.*, 174:847-858, 1991) through their capability of forming stable complexes with MHC molecules or by replacing low affinity peptides from endogenously

filled MHC (Reay, P.A., *et al., The EMBO Journal,* 11:2829-2839, 1992; Fairchild, *et al., Intl. Immunol.,* 5:151-1158, 1993). The formation of stable MHC-peptide complexes could also lead to high avidity interactions with the TCR. It is noteworthy that the recognition of the high affinity variant, 9.4Met, is not blocked by anti-CD4 mAb whereas recognition of the low affinity variant, 9.4Lys or 9.4Arg is completely inhibited. Therefore, the nature of the entire stimulus brought about by various interactions, including co-receptor (e.g. CD4), costimulatory (e.g. CD28) and adhesion molecules, could elicit different T cell effectors with a unique pattern of cytokine secretion. Thus, structural changes in a ligand affecting MHC-binding represent a potential target for immune manipulation of effector function of T cells involved in autoimmune diseases, allergy as well as AIDS, where antigen-specific T cell responses must be redirected into a healthy Th1 or Th2 pattern.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR MODULATING A T-CELL RESPONSE

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE: 26-AUG-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Tumarkin Ph.D., Lisa A.,
(B) REGISTRATION NUMBER: P-38,347
(C) REFERENCE/DOCKET NUMBER: FD3653

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 455-5100
(B) TELEFAX: (619) 455-5110

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

Leu Leu Pro Phe Asn
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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
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(B) LOCATION: 1..17

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Ala

## CLAIMS

1. A method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IFN-$_\gamma$ secreting T cell population, comprising:
   a. preparing an array of variant immunodominant peptides from the antigen; and
   b. selecting from the array, a peptide that has high MHC binding affinity and that induces proliferation of T cells specific for the antigen.

2. The method of claim 1, wherein the T cell population further secretes a lymphokine selected from the group consisting of IL-2 and tumor necrosis factor (TNF).

3. The method of claim 1, wherein the immunodominant peptide is a core peptide.

4. The method of claim 1, wherein induction of T cell proliferation is measured directly.

5. The method of claim 1, wherein induction of T cell proliferation is measured indirectly.

6. The method of claim 1, wherein MHC binding is measured directly.

7. The method of claim 1, wherein MHC binding is measured indirectly.

8. The method of claim 1, wherein step b. is *in vitro*.

9. The method of claim 1, wherein step b. is *in vivo*.

10. The method of claim 1, wherein the IFN-$\gamma$ secreting T cell further mediates delayed type hypersensitivity (DTH).

11. The method of claim 1, wherein the IFN-$\gamma$ secreting T cell further activates macrophages.

12. A method for identifying a T cell modulatory peptide for a specific antigen, wherein the peptide stimulates an IL-4 secreting T cell population, comprising:

a. preparing an array of variant immunodominant peptides from the antigen; and

b. selecting from the array, a peptide that has low MHC binding affinity and that induces proliferation of T cells specific for the antigen.

13. The method of claim 2, wherein the T cell population further secretes a lymphokine selected from the group consisting of IL-5, IL-6, IL-9, IL-10 and IL-13.

14. The method of claim 2, wherein the immunodominant peptide is a core peptide.

15. The method of claim 2, wherein MHC binding is measured directly.

16. The method of claim 2, wherein MHC binding is measured indirectly.

17. The method of claim 2, wherein induction of T cell proliferation is measured directly.

18. The method of claim 2, wherein induction of T cell proliferation is measured indirectly.

19. The method of claim 2, wherein step b. is *in vitro*.

20. The method of claim 2, wherein step b. is *in vivo*.

21. The method of claim 2, wherein the IL-4 secreting T cell further mediates an IgE response.

22. The method of claim 2, wherein the IL-4 secreting T cell further induces B-cell differentiation.

23. A method of modulating a T cell response to a specific antigen in a subject with a T cell disorder, comprising administering to the subject an effective amount of a T cell modulatory peptide for the antigen, wherein the peptide stimulates an IFN-$\gamma$ secreting T cell population.

24. The method of claim 23, wherein the T cell disorder is an allergic disorder.

25. The method of claim 24, wherein the allergic disorder is selected from the group consisting of allergic rhinitis, asthma, atopic dermatitis, and food allergy.

26. The method of claim 23, wherein the T cell disorder is associated with an infection by a helminth.

27. A method of modulating a T cell response to a specific antigen in a subject with a T cell disorder, comprising administering to the subject an effective amount of a T cell modulatory peptide for the antigen, wherein the peptide stimulates an IL-4 secreting T cell population.

28. The method of claim 27, wherein the T cell disorder is an autoimmune disorder.

29. The method of claim 28, wherein the autoimmune disorder is selected from the group consisting of type 1 insulin-dependent diabetes mellitus, adult respiratory distress syndrome, inflammatory bowel disease, dermatitis, meningitis, thrombotic thrombocytopenic purpura, Sjögren's syndrome, encephalitis, uveitic, leukocyte adhesion deficiency, rheumatoid arthritis, rheumatic fever, Reiter's syndrome, psoriatic arthritis, progressive systemic sclerosis, primary biliary cirrhosis, pemphigus, pemphigoid, necrotizing vasculitis, myasthenia gravis, multiple sclerosis, lupus erythematosus, polymyositis, sarcoidosis, granulomatosis, vasculitis, pernicious anemia, CNS inflammatory disorder, antigen-antibody complex mediated diseases, autoimmune haemolytic anemia, Hashimoto's thyroiditis, Graves disease, habitual spontaneous abortions, Reynard's syndrome, glomerulonephritis, dermatomyositis, chronic active hepatitis, celiac disease, autoimmune complications of AIDS, atrophic gastritis, ankylosing spondylitis and Addison's disease.

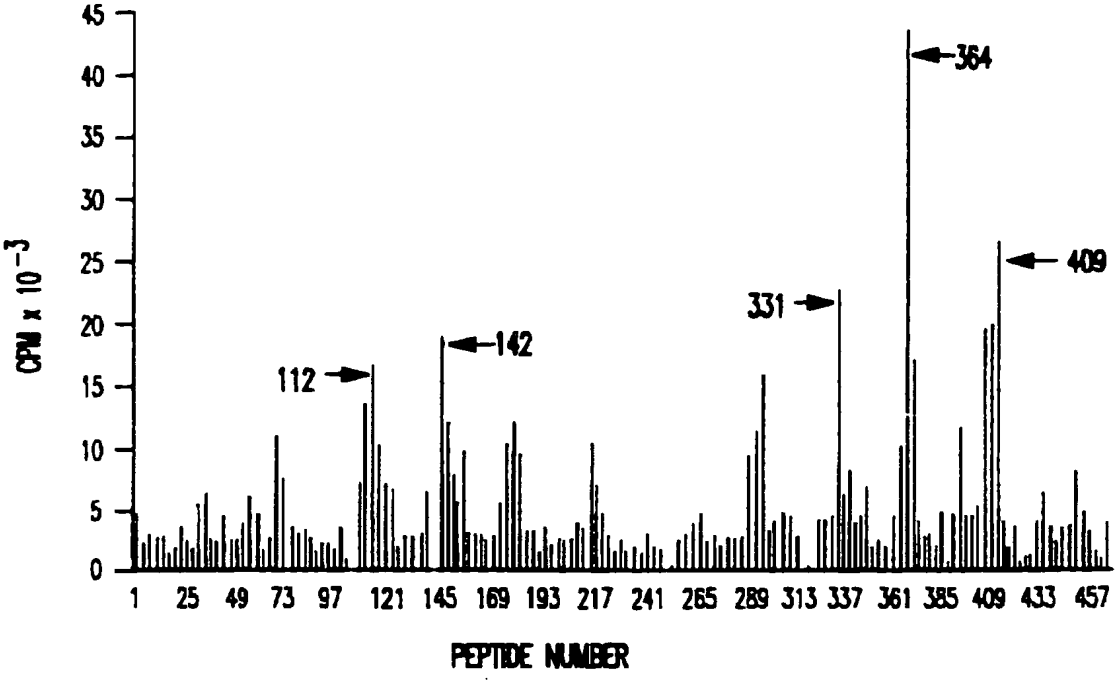
30. The method of claim 27, wherein the T cell disorder is associated with an infection with an organism selected from the group consisting of a bacteria, a virus and a protozoa.

31. A T cell modulatory synthetic peptide having an amino acid sequence consisting of ASQXRPSQR, wherein X is selected from the group consisting of glutamic acid, glutamine, alanine, valine, tyrosine and methionine.

32. A polynucleotide sequence that encodes the peptide of claim 31.

33. A T cell modulatory synthetic peptide having an amino acid sequence consisting of YSDGSCTQRASEAHASLLPFN.

34. A polynucleotide sequence that encodes the peptide of claim 33.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PEPTIDE NUMBER

FIG. IA

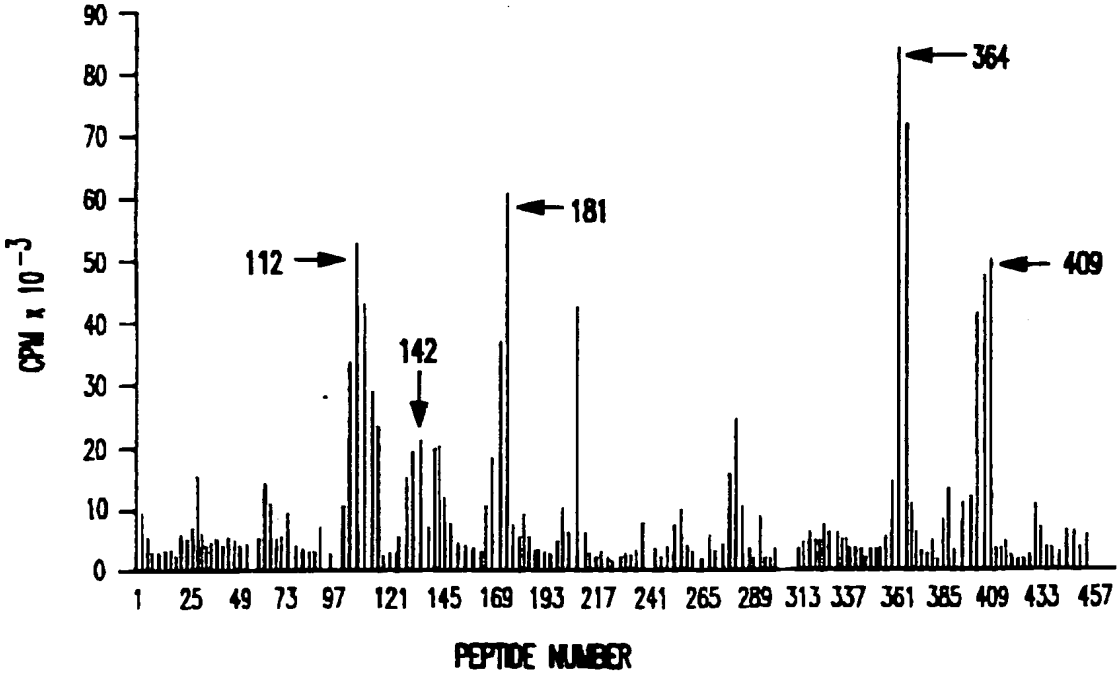
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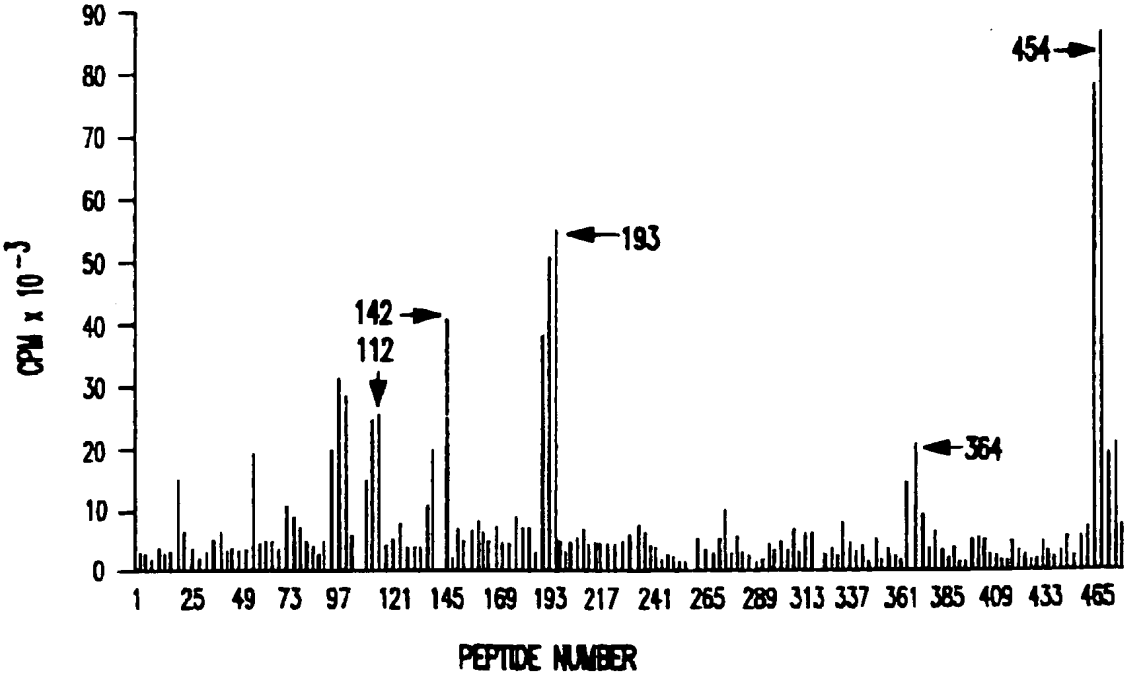
FIG. IB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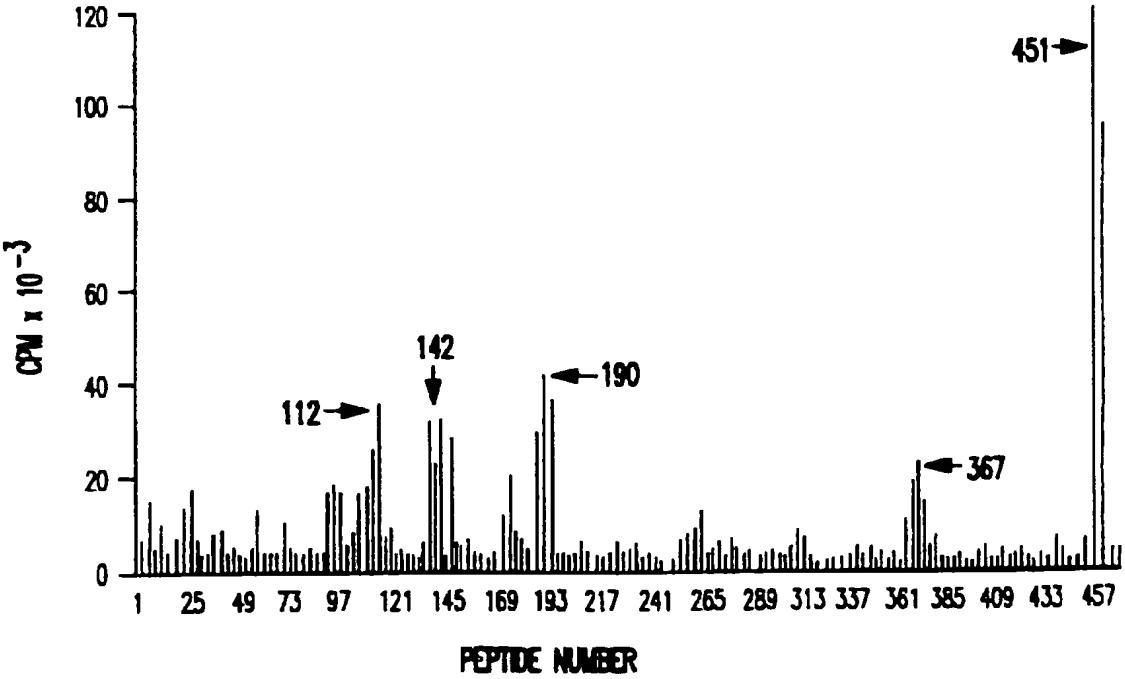
1 25 49 73 97 121 145 169 193 217 241 265 289 313 337 361 385 409 433 465
PEPTIDE NUMBER

FIG. IC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60
40
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0
CPM x $10^{-3}$
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142
190
112
367
1 25 49 73 97 121 145 169 193 217 241 265 289 313 337 361 385 409 433 457
PEPTIDE NUMBER

FIG. ID

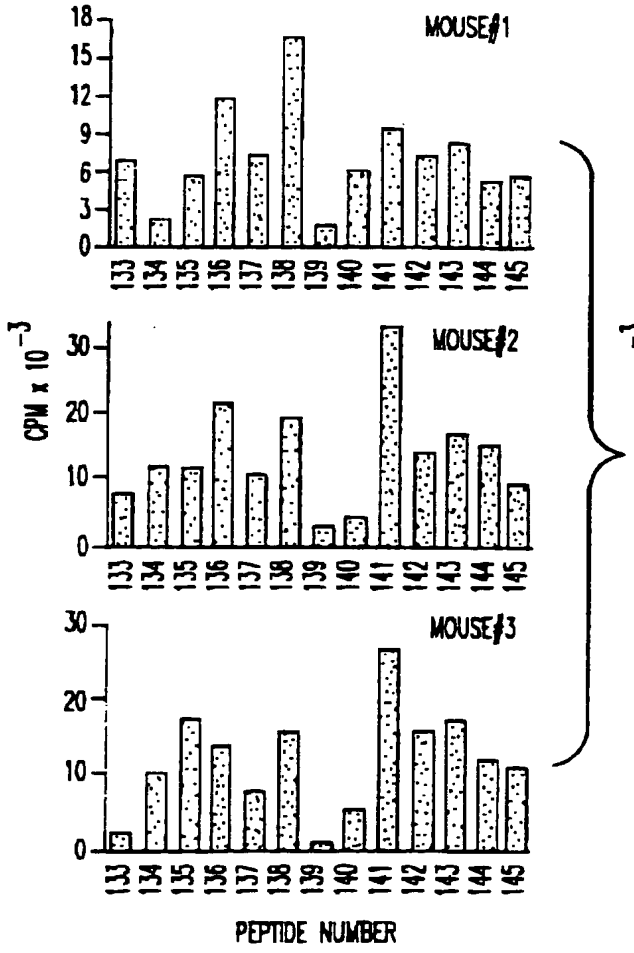

MOUSE#1
MOUSE#2
MOUSE#3
CPM x $10^{-3}$
PEPTIDE NUMBER

FIG. 2A

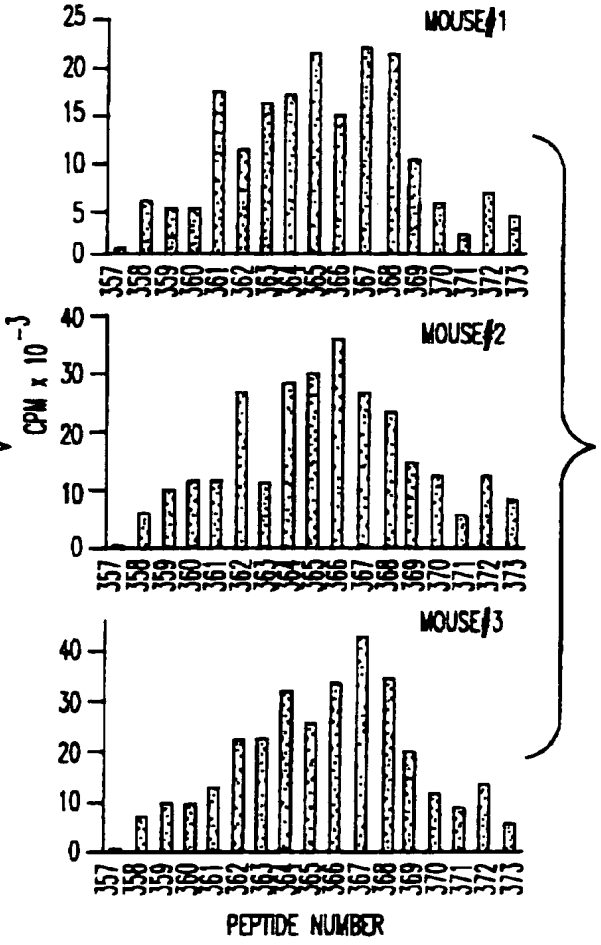

MOUSE#1
MOUSE#2
MOUSE#3
CPM x $10^{-3}$
PEPTIDE NUMBER

FIG. 2B

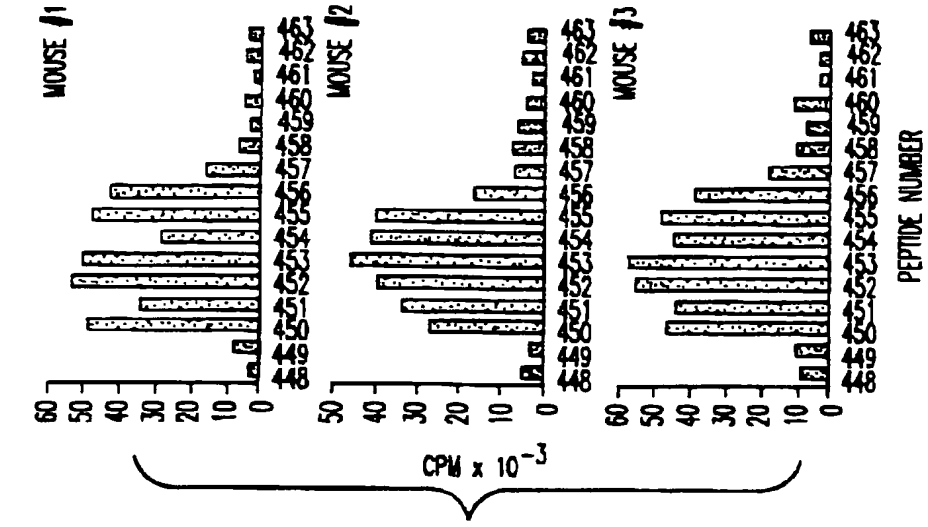



FIG. 3C

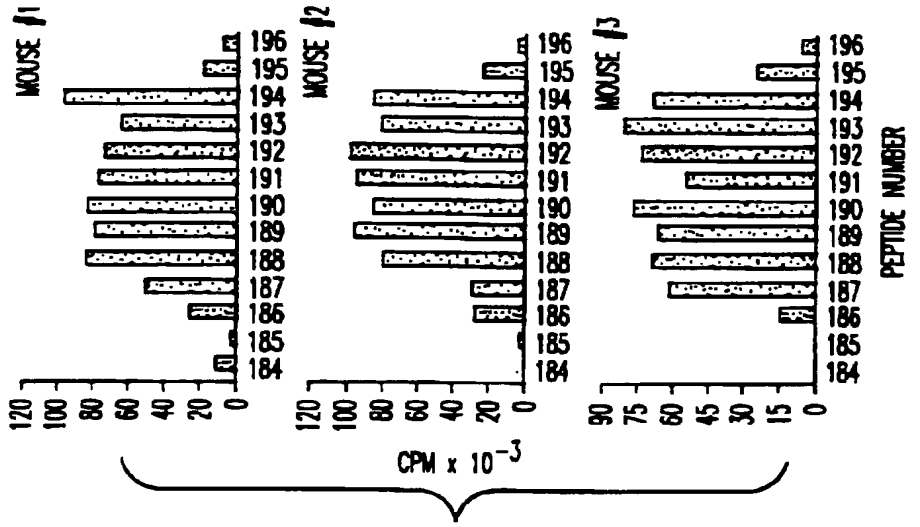



FIG. 3B

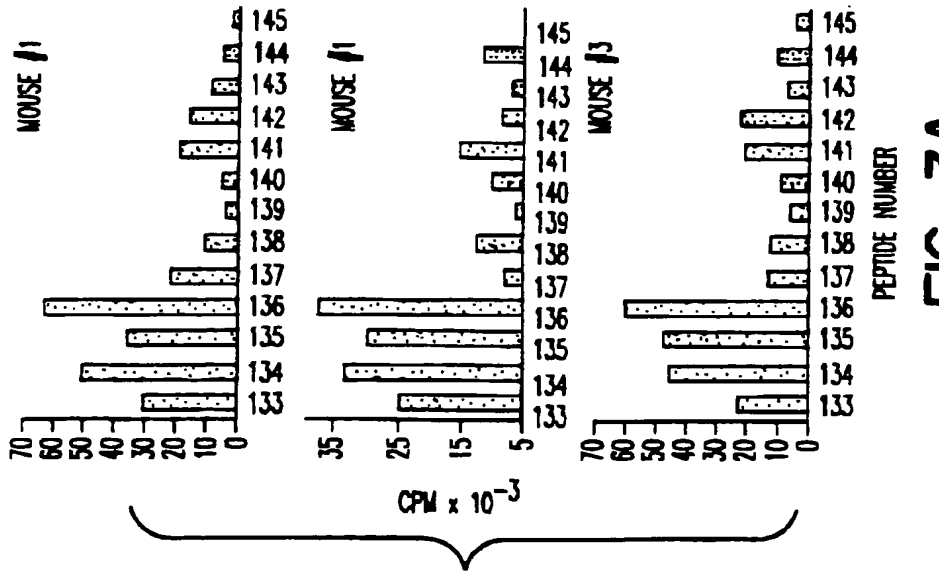



FIG. 3A

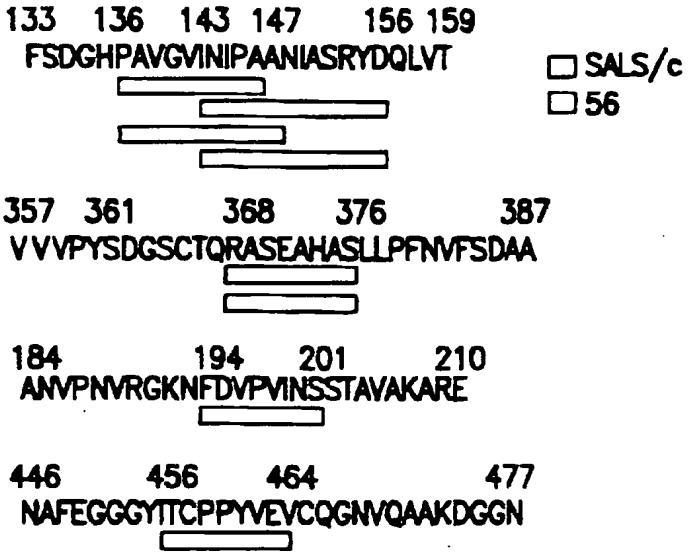

133 136 143 147 156 159
FSDGHPAVGVINIPAANIASRYDQLVT
SALS/c
56
357 361 368 376 387
VVVPYSDGSCTQRASEAHASLLPFNVFSDAA
184 194 201 210
ANVPNVRGKNFDVPVINSSTAVAKARE
446 456 464 477
NAFEGGGYITCPPYVEVCQGNVQAAKDGGN

FIG. 4

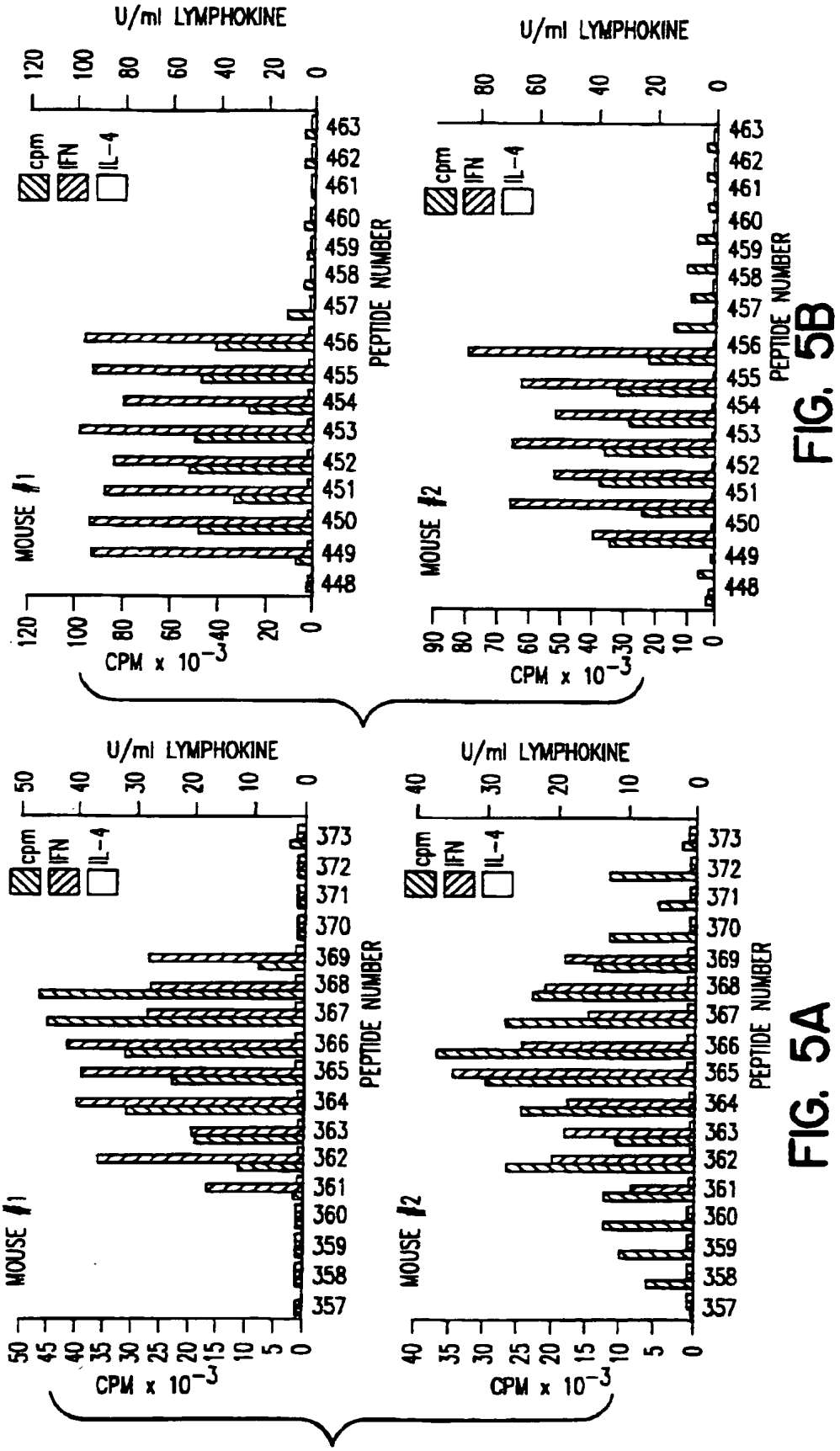

U/ml LYMPHOKINE
cpm
IFN
IL-4
PEPTIDE NUMBER
MOUSE #1
MOUSE #2
CPM x $10^{-3}$

FIG. 5B

FIG. 5A

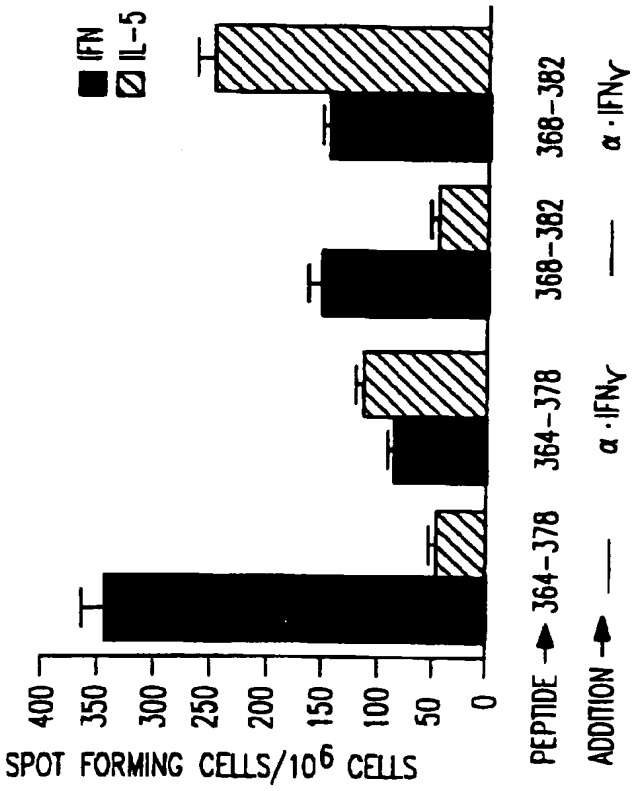

IFN
IL-5
400
350
300
250
200
150
100
50
0
SPOT FORMING CELLS/10^6 CELLS
PEPTIDE → 364-378 364-378 368-382 368-382
ADDITION → — α·IFNγ — α·IFNγ

FIG. 6A

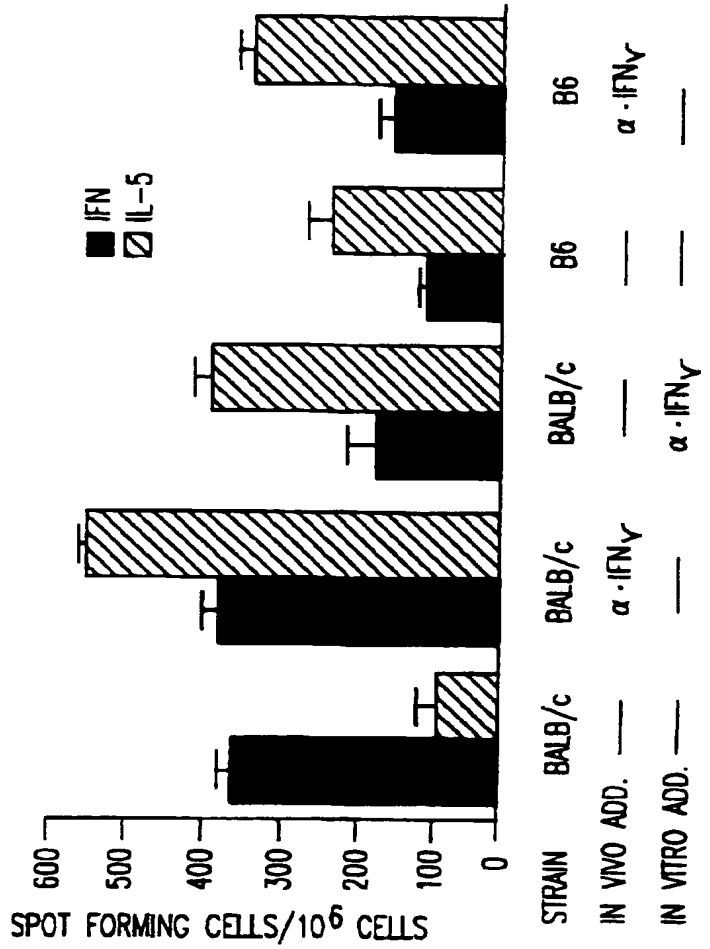

IFN
IL-5
600
500
400
300
200
100
0
SPOT FORMING CELLS/$10^6$ CELLS
STRAIN
IN VIVO ADD.
IN VITRO ADD.
BALB/c
BALB/c
BALB/c
B6
B6
B6
—
α·IFNγ
—
—
α·IFNγ
—
—
α·IFNγ
—

FIG. 6B

FIG. 7A

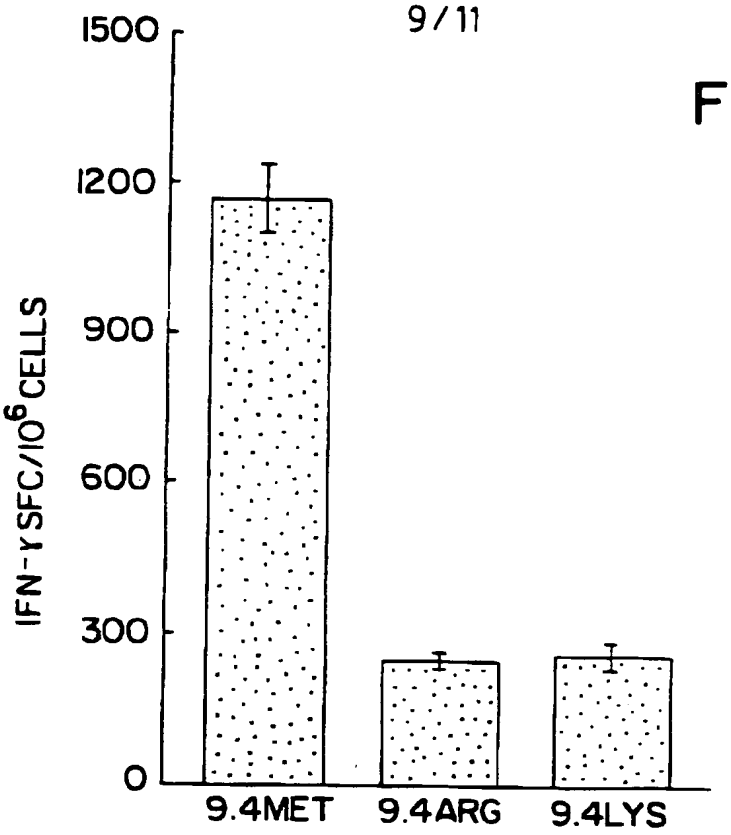

1500
1200
900
600
300
0
IFN-γ SFC/10^6 CELLS
9.4MET
9.4ARG
9.4LYS

FIG. 7B

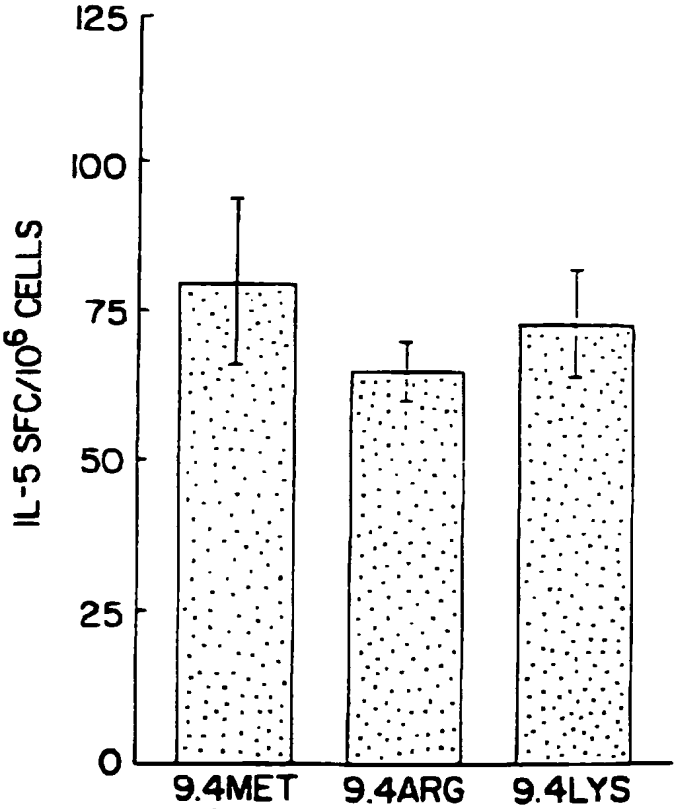

125
100
75
50
25
0
IL-5 SFC/10^6 CELLS
9.4MET
9.4ARG
9.4LYS

FIG. 9A

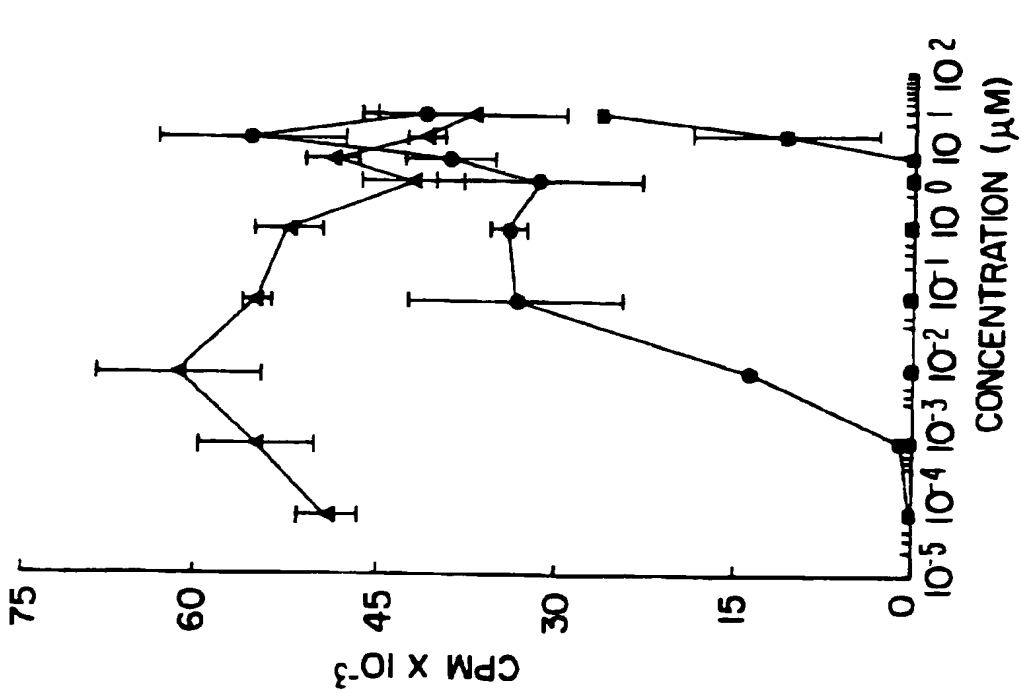

CPM X 10⁻³
75
60
45
30
15
0
10⁻⁵ 10⁻⁴ 10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10²
CONCENTRATION (μM)

FIG. 8

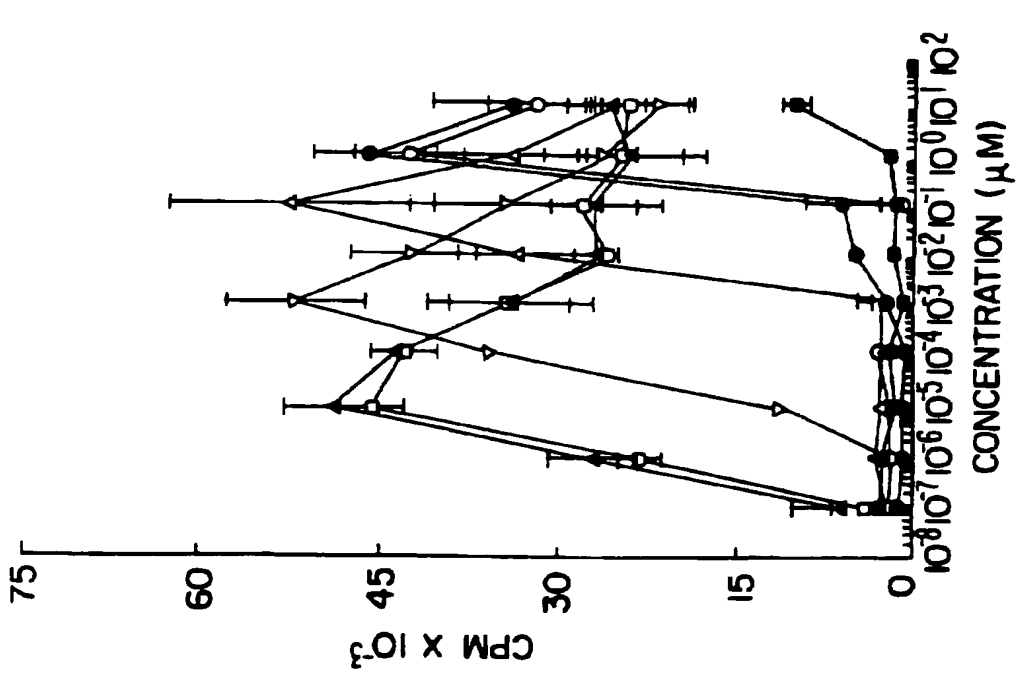

CPM X 10⁻³
75
60
45
30
15
0
10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10²
CONCENTRATION (μM)

FIG. 9C

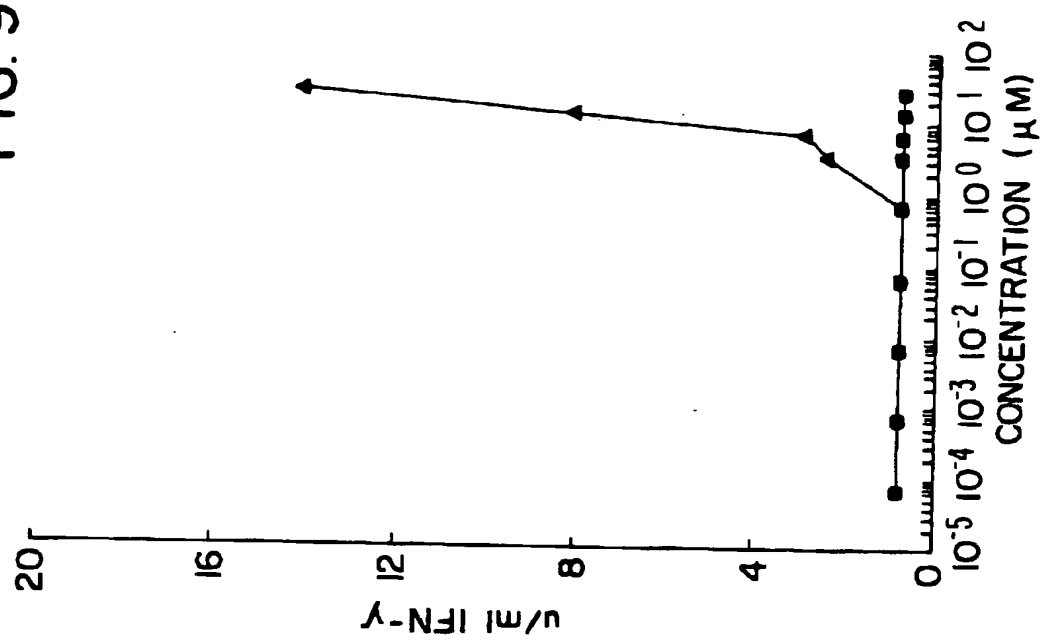
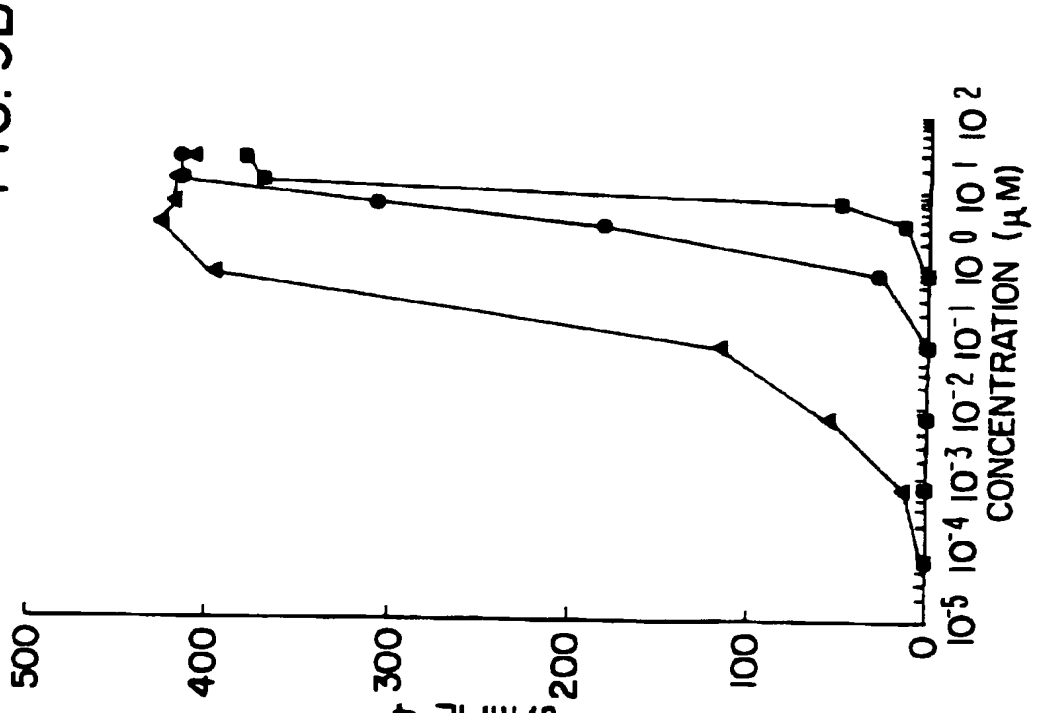

u/ml IFN-γ
20
16
12
8
4
0
$10^{-5}$ $10^{-4}$ $10^{-3}$ $10^{-2}$ $10^{-1}$ $10^{0}$ $10^{1}$ $10^{2}$
CONCENTRATION (μM)

FIG. 9B


u/ml IL-4
500
400
300
200
100
0
$10^{-5}$ $10^{-4}$ $10^{-3}$ $10^{-2}$ $10^{-1}$ $10^{0}$ $10^{1}$ $10^{2}$
CONCENTRATION (μM)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09795

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/185.1, 191.1; 435/7.24, 7.8; 436/501, 506, 513; 514/13, 15; 530/326, 328; 930/DIG 802, DIG 821

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Cas Online, GeneSeq, PIR, Swiss Prot for sequence of claim 31.
GeneSeq, PIR, Swiss Prot for sequence of claim 33

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO, A, 94/01560 (GRIFFITH ET AL.) 20 January 1994. See page 14, line 25-page 15, line 29, particularly page 15, line 20. | 23-25 |
| X<br>---<br>A | Proceedings of the National Academy of Sciences USA, Vol. 87, issued February 1990, Kumar et al, "Amino acid variations at a single residue in an autoimmune peptide profoundly affect its properties: T-cell activation, major histocompatibility complex binding, and ability to block experimental allergic encephalomyelitis", pages 1337-1341. See abstract, page 1338, col. 1 and page 1340, cols. 1-2. | 31<br>---<br>12-22, 27-30 |
| A | US, A, 5,200,320 (SETTE ET AL.) 06 April 1993. See abstract. | 3-8, 14-19 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 01 FEBRUARY 1995 | 13 FEB 1995 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | DAVID SAUNDERS, PRIMARY EXAMINER |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Journal of Experimental Medicine, Vol. 174, issued October 1991, SOLOWAY ET AL, "Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype", pages 847-858. See Summary and page 855, col. 1. | 1-10 and 23-26 |
| A | Science, Vol. 260, issued 14 May 1993, LANZAVECCHIA, "Identifying strategies for immune intervention", pages 937-944. See abstract, page 938, cols. 2-3; page 942, col. 1. | 1-30 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09795

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-31 and 33

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09795

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/08, 38/16, 39/00, 39/008; C07K 7/06, 14/44; G01N 33/564, 33/566

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/185.1, 191.1; 435/7.24, 7.8; 436/501, 506, 513; 514/13, 15; 530/326, 328; 930/DIG 802, DIG 821

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-11, and 23-26, drawn to the identification of T-cell modulatory peptides stimulating IFN-gamma secreting T-cells, a peptide, and body treatment methods.

II. Claims 12-22, and 27-30, drawn to the identification of T-cell modulatory peptides stimulating IL-4 secreting T-cells, a peptide, and body treatment methods (Note the Examiner assumes that claims 13-22 are intended to depend from claim 12, rather than 2).

III. Claim 31, drawn to a peptide from myelin basic protein.

IV. Claim 32, drawn to a polynucleotide encoding a peptide from myelin basic protein.

V. Claim 33, drawn to a PSP peptide.

VI. Claim 34, drawn to a polynucleotide encoding a PSP peptide.

The methods of Groups I and II identify T-cell populations that differ in their secretion characteristics, and the methods do not involve common steps, i.e. Group I includes a step of selecting for high MHC binding affinity, while Group II includes a step of selecting for low MHC binding affinity.

The peptides of Groups III and V are not clearly related to either of the selection methods of Groups I or II. Also the two peptides of Groups III and V have no common structural core, i.e. no common amino acid sequence. Groups III and V thus have no special technical feature to provide for unity of invention.

The peptides of Groups III and V are not related, respectively, to the polynucleotides of Groups IV and VI, because a peptide and a polynucleotide contain no common structural core. Also the polynucleotides of Groups IV and VI contain no common structural core, i.e. no common nucleotide sequence. Groups IV and VI thus have no special technical feature to provide for unity of invention.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.